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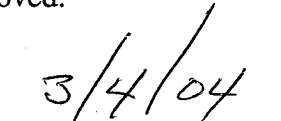
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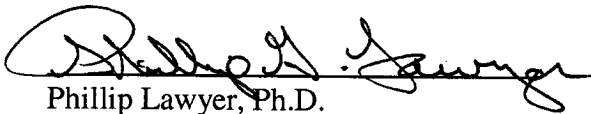
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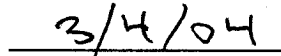
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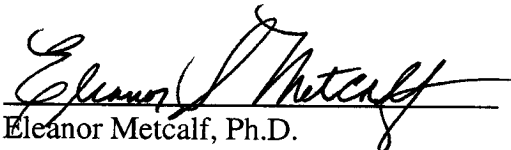
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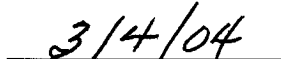
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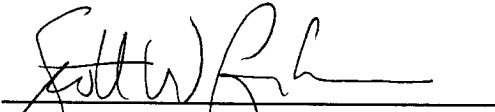
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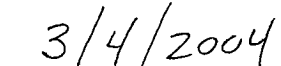
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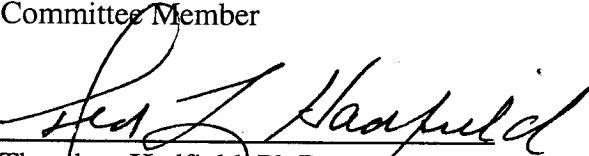
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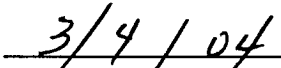
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## Abstract

Evidence incriminating phlebotomine sand flies as vectors of *Bartonella bacilliformis*, which causes bartonellosis, has not been proven beyond doubt. Our research was designed to strengthen this hypothesis. In addition, we developed a Real-Time PCR method capable of detecting as little as 100fg of *Bartonella* DNA in sand flies. Twelve primers were designed using TaqMan Probe/Primer Data and tested.

*Lutzomyia verrucarum* sand flies were collected from an endemic focus of bartonellosis in and around Caraz, Ancash, Peru. Unfed female sand flies were pooled in groups of 5 for DNA extraction. Of 472 pools assayed, 13 tested positive (2.75%). The sensitivity of the test was 100% and specificity was 91.11% based on assays of known samples. We conclude that this method has the necessary sensitivity and specificity to detect *B. bacilliformis* infection in wild-caught sand flies enabling us to determine the true infection sand fly rates in an endemic area of Peru.

**Detection of *Bartonella bacilliformis* by Real-Time PCR in Naturally  
Infected Sand Flies**

By

Sofia Romero

Thesis submitted to the Faculty of the  
Department of Preventive Medicine and Biometrics  
Uniformed Services University of the Health Sciences  
In partial fulfillment of the  
requirements for the degree of  
Master of Science 2004

## Research Objectives

The aim of this study was to develop a rapid and sensitive real-time PCR assay, that would allow detection and quantification of *Bartonella bacilliformis* in field and experimental samples of vector sand flies. Such diagnostic tools are needed in field and laboratory studies for the analysis of the epidemiology and transmission dynamics of bartonellosis. By learning more about the cycles of natural infection in sand flies, seasonal variations and the mechanism of transmission by bite of the sand flies, resources can be better directed to help prevent the transmission of *Bartonella bacilliformis* to humans.

This new procedure was elaborated in order to detect small quantities of *Bartonella bacilliformis* DNA in samples. This more sensitive test will more accurately reflect infectivity rates in field populations of sand flies, enabling a closer approximation of the true risk of human infection in endemic areas.

To my family, friends and teachers for all their  
support and guidance through all my studies.

## Table of Contents

Approval Sheet.....	i
Copyright Statement.....	ii
Abstract.....	iii
Title Page.....	iv
Objectives .....	v
Dedication.....	vi
Table of contents.....	vii
List of Tables.....	x
List of Figures.....	x
Background.....	1
Overview of Bartonellosis.....	1
History.....	2
Clinical Features.....	2
Oroya Fever.....	3
Verruga Peruana.....	3
Asymptomatic Infection.....	4
Treatment.....	4
Epidemiological Studies.....	5
Diagnosis.....	6
Vector Overview.....	7
History.....	7
Description and Biology of Phlebotomine Sand Flies.....	8

Molecular Data.....	9
Distribution of Phlebotomine Sand Flies.....	9
Vector Incrimination.....	11
Prevention and Control .....	12
Principles of Real-Time PCR.....	13
PCR Basics.....	13
Analysis.....	14
Real-Time PCR.....	15
Annealing Temperature and Primer Design .....	16
Probe Selection TaqMan® Probe.....	16
Citrate sy18nthase gene: <i>gltA</i> .....	17
Materials and Methods .....	18
Study Site.....	18
Sand Fly Collection.....	18
Sand Fly DNA Isolation Using Qiagen Dneasy Tissue Kit.....	18
DNA Extraction of <i>Bartonella</i> sp. Strains with Qiagen DNA Kit.....	20
Conventional PCR.....	21
Electrophoresis for DNA Detection .....	22
Real-Time PCR Using the LightCycler thermocycler.....	22
Primer design.....	22
Basic Real-time reaction set up.....	23
Optimization of primer concentration.....	24

Optimization of probe concentration.....	25
Limit of Detection Quantification.....	25
Specificity.....	26
Analysis of the amplicons with Agilent.....	27
Sequencing of the amplicons.....	27
Statistical analysis.....	27
Results.....	28
Discussion.....	46
Conclusion.....	51
Appendices.....	53
Bibliography.....	79

## List of Tables.

Table 1. *Bartonella bacilliformis* strains used from GeneBank.

Table 2. PCR reaction set up

Table 3. Primer optimization check board.

Table 4. Conventional PCR results.

Table 5. *Bartonella bacilliformis* strains used as controls.

Table 6. Distribution of results by houses.

Table 7. Field sample results.

## List of Figures

Figure 1. Correlation of bartonellosis cases with *Lutzomyia verrucarum* distribution.

Figure 2. Map of study area showing towns of Choquechaca, Cullashpampa,

Yuracoto and Caraz. Red line represents the main road to the area.

Figure 3. The photo to the left is a thick smear of 5-day *Bartonella bacilliformis* culture.

The photo at the right side shows a thin smear of the same culture. Slides are stained with Giemsa.

Figure 4. Test with USUHS reagents and primers (samples 1 - 8) compared to AFIP reagents and USUHS primers (samples 9 - 16) in the LightCycler with a 3 step PCR: 30-60-60. The graphic shows that AFIP reagents worked better.

Figure 5. Results of optimization experiment testing primers BBF1-BBR1. Tube 12 is best combination, concentration 250 forward, 500 reverse.

Figure 6. Optimization of primer concentration for primers BBF2 and BBR2.

Figure 7. Optimization of primer concentration for primers BBF3 and BBR3. The primer concentration selected was 250nM for the forward and 500nM for the reverse.

Figure 8. Probe optimization of BART-1 was 2 $\mu$ M.

Figure 9. BART-MGB screening showed no amplification to some of the positive controls.

Figure 10. Pools of 10 negative *Lutzomyia verrucarum* show amplification, the NTC control was negative.

Figure 11. The four first sets of new primers were screened in one test.

Figure 12. The melting temperature of the positive samples was 83°C and the negative pool was 87°C using primer Set1.

Figure 13. The melting temperature of the positive samples was 84°C and the negative pool was 86°C using primer Set2.

Figure 14. The melting temperature of the positive samples was 82°C and the negative pool was 84°C using primer Set3, NTC had no amplification.

Figure 15. The melting temperature of the positive samples was 84°C and the negative pool was 82.5°C using primer Set4.

Figure 16. Last three sets of primers showed less fluorescence.

Figure 17. The graph shows the melting temperature of the positive samples was 83°C and the negative pool was 86°C using primer Set5.

Figure 18. The melting temperature of the positive samples using primer Set4 was 82°C, the negative pool showed double peaks of 79°C and 85°C.

Figure 19. The graph shows the melting temperature of the positive samples was 83°C and the negative pool showed 2 peaks of 79°C was 87°C using primer Set7.

Figure 20. Gel electrophoresis showed that all the primers amplified multiple bands for the negative sand fly pool.

Figure 21. Primer Set 2 had the same band pattern for the NTC, and the negative sand fly pool and no 75bp band.

Figure 22. Tube 8 with 500nM for the forward and 250nM for the reverse had the lowest CT and the highest fluorescence.

Figure 23. Tube 12 with 250nM for the forward and 500nM for the reverse was selected as the optimal primer dilution.

Figure 24. Gel electrophoresis of field samples was tested using Set 2 primers with Sybr Green.

Figure 25. New probes B2-203 and BB3-543 were screened at different concentrations along with some field samples.

Figure 26. Optimization of Probe BB2-203 showed that working stock of 2 $\mu$ M gave a higher fluorescence and lower CT's.

Figure 27. Optimization of Probe BB3-543 showed that working stock of 1 $\mu$ M gives a higher fluorescence and lower CT's.

Figure 28. Limit of detection quantification was positive up to 10fg/5 $\mu$ l dilutions.

Figure 29. The LOD was 100fg/5 $\mu$ l when using probe BB2-203.

Figure 30. Specificity test for Probe BB2-203.

Figure 31. *Bartonella* strains and "Negative" pools

Figure 32. Strain controls, different volumes of low infected red blood cells and P.L.'s blood were amplified.

Figure 33. Sand flies genes were not cross-reacting with the test.

Figure 34. Conventional PCR using 781P, 1137N primers showed a 379bp band in the positive samples.

Figure 35. Few field samples gave high positive; many samples with CT readings were possibly negative, due to the low fluorescence.

Figure 36. Primers and probes distributed in the citrate synthase gene.

Figure 37. Season distribution of all the pools tested in four towns of Peru. Shaded area represents positive PCR pools.

Figure 38. Season distribution of infected pools tested in House A. Shaded area represents positive pools to PCR.

Figure 39. Season distribution comparison of infected pools in positive houses. Positive pools were found in all houses in samples of March 2000.

## BACKGROUND

### Overview of Bartonellosis

Bartonellosis, also known as Verruga Peruana and Oroya fever, is an infectious disease caused by Gram-negative pleomorphic bacilli of the  $\alpha$ -proteobacteria called *Bartonella bacilliformis*. This life threatening, emerging infectious disease is found commonly in medically-underserved communities in the Andes Mountains regions of Peru, Ecuador and Colombia (Alexander, 1995). This disease often presents in two phases. The first phase is an acute febrile disease (Oroya Fever) that can be fatal if untreated. The second phase is characterized by cutaneous lesions called hemangiomas that regress and heal over several months without leaving a scar. This chronic eruptive phase or verrucous phase is known as Verruga Peruana.

Observational and experimental data suggest that bartonellosis is vector-borne. The disease usually occurs at elevations between 800 and 2800 meters, and its distribution pattern corresponds to that of certain *Lutzomyia* sand fly species. Recent outbreaks have occurred at over 3000 meters above sea level (Ellis et al. 1999, Kosek et al. 2000). Bartonellosis affects an endemic population of almost 2 million people (MINSA web page Peruvian Ministry of Health). Heavier rains associated with documented El Nino events correlate with an increase of cases and disease severity, making this natural factor a potential cause of epidemics. Increasing national and international travel may also spread the disease to non-endemic areas (Caceres-Rios et al. 1995, Tejada et al. 2003).

To date no non-human animal reservoir has been identified and humans are the only apparent natural reservoirs. The mode of transmission, therefore, is thought to be

human-sand fly-human. The populations at greatest risk are children less than 10 years old and recent immigrants to an endemic area (Maguiña 1998).

## **History**

Bartonellosis has caused debilitating illness and death since pre-Inca times. Evidence of the antiquity of bartonellosis includes the reproduction of verruga found on ceramic statuettes made by pre-Columbian Indians (Kosek et al. 2000).

The acute phase, called Oroya fever, was described during an outbreak in 1870 associated with the construction of a railroad from Lima to La Oroya. Over 7000 workers died which caused much concern in the local and scientific community. Because of the large number of patients, many valuable observations were recorded (Pachas 2000).

Bartonellosis is also called Carrion's disease after Daniel Alcides Carrion, a medical student who, in 1885, proved that Oroya fever and Verruga Peruana are phases of the same disease. After inoculating himself with blood from a verrucous lesion, he developed the acute form of the disease and died 21 days later (Pachas 2000). Daniel Alcides Carrion gave his life pursuing medical knowledge. He is a martyr in Peru and a national day of medicine commemorates his sacrifice.

## **Clinical Features**

The incubation period of bartonellosis is thought to be three to ten months. Clinically, *Bartonella bacilliformis* infection presents in two different phases: an acute phase manifested by a hemolytic anemia and an eruptive rash phase called Verruga Peruana. Asymptomatic infections also occur. According to several studies, the

mortality rate in untreated patients during the acute phase can reach 90%. Concomitant infections during the acute phase, some of which are opportunistic including typhoid fever, tuberculosis reactivation, pneumocystis pneumonia and others, account for most of this additional mortality (Maguiña 1998).

### **Oroya Fever**

The acute hemolytic disease may present with fever and protean symptoms and is characterized by mild to severe anemia. During the acute stage, up to 100% of the red blood cells may be parasitized and 80% lysed. *Bartonella bacilliformis* is the only bacterium that is known to invade human red blood cells. The organism enters the erythrocyte where it proliferates, and later it lyses the cell in order to invade the next erythrocyte. For many years, researchers thought the bacilli did not penetrate but adhered to the cell wall of the red blood cells. However in 1969, Takano, using electron microscopy, proved that the bacteria did indeed penetrate the erythrocytes (Cuadra and Takano 1969). Some preliminary evidence exists which suggests that *B. bacilliformis* interacts with multiple surface-exposed proteins of the human erythrocytes that function as receptors for the penetration (Buckles and McGinnis 2000).

### **Verruga Peruana**

The eruptive or verrucous phase is called Verruga Peruana. It usually occurs four to eight weeks after the symptoms of Oroya fever have resolved and is characterized by hemangiomas (benign vascular tumors). This stage presents as a variety of lesions and can occur regardless of prior antibiotic treatment (Velho et al. 2003). The lesions, which frequently bleed spontaneously, are apparently the result of partial immunity to the

infection (Alexander 1995). During this phase, the bacteria invade vascular endothelial cells and subsequently stimulate the formation of new blood vessels (Maguiña 1998). The eruptive or verruga phase, although not frequently severe, can last several months. Normally the lesions do not leave scars.

There are three different types of verrucous lesions. Miliar lesions are very small and usually disseminated. They localize in the papillar dermis. Mular verruga are large lesions and can extend all the way to the hypodermis. Early histological sections show proliferation of endothelial cells and macrophages. The number of mitoses is variable, and mitosis number correlates with the size of the lesion. In some cases, the lesions are numerous and are associated with atypical cells. Mular lesions give a histological image similar to a malignant neoformation. Lymphocytes, mastocytes and plasmatic cells are rarely found. The evolution of the lesion usually starts with tenderness and ends with a final reabsorption of the vascular lesion (Maguiña 1998). Finally, nodular lesions are subcutaneous, usually asymptomatic, and most of these lesions will heal without treatment in three to six months (Maguiña 1998).

### **Asymptomatic Infection**

There is also an asymptomatic intermediate period between the acute and chronic phase that varies in its duration. This stage is thought to be the most important in the transmission cycle, and asymptomatic carriers are believed to be reservoirs of the disease (Maguiña 1998).

### **Treatment**

*Bartonella bacilliformis* is highly susceptible to antibiotics. In Peru, the Ministry of Health (MINSA) uses chloramphenicol to treat adult patients with acute infections

(MINSA web page). Ampicillin is given to children, along with blood transfusions for severe anemia. For verruga patients, rifampicin and streptomycin are used. Treatment varies, depending on the location, *i.e.* whether it is in an endemic or non-endemic area. The Ministry of Health divides the country into Health regions, and each has different treatment schemes. Antibiotic regimens have been determined empirically. A recent study, using four strains of *Bartonella bacilliformis*, showed that this organism displays high in vitro susceptibility to most antibiotics (Sobraques et al. 1999). Resistance to traditional treatment is now more common, especially in the case of the recent outbreaks (Personal communication, Dr. Manuel Montoya). New treatment schemes must be tried in order to prevent resistance. The mechanism of resistance has not yet been delineated (Barbian and Minnick 2000).

### **Epidemiological Studies**

Epidemiological data on bartonellosis are very limited due to the remote location of outbreaks. Retrospective epidemiological surveys are usually based on information obtained from hospital and clinical records, correlated with histopathology and serology. Such methods tend to underestimate the spectrum of the disease symptoms and overestimate the real morbidity and mortality (Kosek et al. 2000). In areas where *B. bacilliformis* is endemic, the prevalence of infection is around 45% (Chamberlin et al 2000). A prospective cohort study conducted in Caraz found an incidence of 12.7/100 person-years (Chamberlin et al. 2002).

Even though clinical data support the important role of the host response in the clinical outcome of infection (Maguiña 1998), little is known about the immunological responses to *Bartonella bacilliformis*. A few sporadic and unpublished studies have

analyzed immune responses to this disease. However, none were conducted prospectively or carried out using modern immunological techniques (Maguiña 1998).

## **Diagnosis**

Diagnosis of bartonellosis is difficult, and few laboratories work on *Bartonella*. Diagnosis by blood smears requires experience and good staining techniques in order to find the intraerythrocytic bacteria. Possible infection is indicated by the number of reticulocytes observed; in bartonellosis, up to 50% reticulocytosis is frequently found. The bacilli are slow growing in culture and require special techniques, which include the culture of samples in biphasic medium; blood-based agar with an enriched broth (Malqui et al. 2000). These bacteria grow best at 28°C in biphasic medium supplemented with 5-10% whole sheep blood and an additional 5 ml of enriched medium such as RPMI that provides the humidity required for long incubations. The cultures should be kept and monitored for at least eight weeks to insure a good recovery rate. There is no selective medium available for this bacterium; hence, sterility measures are essential to avoid contamination. Types of specimens that may be used to help confirm a diagnosis in suspected patients are sera which can be used for serological tests such as Indirect fluorescence assay (IFA) or Western blot (Malqui et al. 2000, Gray et al. 1990, Knobloch et al. 1985), citrated blood for culture, blood for Polymerase chain reaction (PCR), and biopsies or aspirates of verruga lesions (Chamberlin et al 2000, Knobloch et al. 1985). Transportation of samples is an important issue because the arrival of samples at a reference laboratory may be delayed.

PCR has been an essential tool for recent discoveries on *Bartonella*. Genes targeted so far include the 16S-23S intergenic spacer region, 16S rRNA, citrate synthase

(*gltA*), *IalB*, *groEL*, *nlpD/lppB* L-isoaspartyl methyltransferase, *ddlB*, *ftsQ* and *ftsA* genes upstream of *ftsZ*, flagellin (*flaA*) and *ribC* (Barbian and Minnick 2000, Padmalayam et al. 2003, Coleman and Minnick 2001, Ellis et al. 1999, Battisti and Minnick 1999).

## Vector Overview

### History

Entomologists suspect the vectors of *B. bacilliformis* to be nocturnal-feeding phlebotomine sand flies. The first written account known to implicate phlebotomine sand flies as vectors of Carrion's disease and cutaneous leishmaniasis in Peru was published by Cosme Bueno in 1764, in a newspaper in Lima, Peru. These findings were confirmed in an article in 1975 in *Science* (Herrer and Christensen 1975). Bueno's report precedes other publications implicating sand flies in the transmission of human pathogens by nearly a century and a half.

In 1913, Townsend reported that *Phlebotomus verrucarum* (Now *Lutzomyia verrucarum*) was the vector of bartonellosis. In his work, he eliminated any other possible vector such as mites, kissing bugs and other arthropods (Hertig 1942). Other scientists, Noguchi, Shannon, Battistini, Herrer and Hertig later corroborated this (Battistini 1925, Hertig 1942).

In 1925, Battistini reported that he had experimentally transmitted bartonellosis to rhesus monkeys by inoculating them intradermally with a homogenate of wild-caught sand flies collected from the Verruga zone. Because this experiment did not prove transmission by sand fly bite, he later infected rhesus monkeys with suspected infected sand flies by releasing them in a cage with the monkeys. Although this study was the

first sand fly experiment in which the disease was transmitted by means other than inoculation, the sand flies were not observed, and it was not determined whether transmission was by sand fly bite. (Battistini 1925)

In 1937, Hertig and associates began a series of feeding experiments in which wild-caught sand flies were either released into small cages with a rhesus monkey or placed in small feeding cages and strapped to the monkey's shaved belly. Several monkeys became infected to *B. bacilliformis* as determined by blood culture, but cultures of sand flies were not prepared; therefore it was not possible to determine if the sand flies were infected. Furthermore, slight uncertainty remained as to whether transmission was by bite or by some other means, such as by sand fly defecation. Later, Hertig and associates isolated what they said was *B. bacilliformis* from the proboscis of each of two "*Phlebotomus verrucarum*" collected from an epidemic focus at Huinco in the Santa Eulalia Valley. Because culture methods were not sufficiently refined to distinguish between *B. bacilliformis* and other *Bartonella* species, there is still a question as to whether this bacillus was the same pathogen that causes disease in humans (Hertig 1942).

### **Description and Biology of Phlebotomine Sand Flies**

Phlebotomine sand flies belong to the Order Diptera, Family Psychodidae. They are small (2-4 mm in length), delicate flies with long, thin legs and narrow, pointed wings with parallel venation; the body is covered with hair-like setae. At rest, the wings held upright at about 60°, forming a "V". These tiny arthropods have a limited flight range. Basically they do not fly, they hop. They prefer to rest in dark, humid places like animal burrows, tree holes, on or under tree bark, on undersides of leaves, under rocks, in rock crevices, etc. Due to their small size, they are able to pass through the mesh of a standard

mosquito net. Most human-biters feed at dusk and during the evening hours, but some species will bite during the day as well if they are disturbed in their resting site. Some anthropophilic sand flies are such as *Lutzomyia verrucarum*, the suspected vector in this study, are endophagic and readily enter human dwellings, where they bite the occupants and leave or rest inside (Hertig 1942, Lawyer and Perkins 2000, Gupta et al. 2003).

### **Molecular Data**

Very little molecular data on *Lutzomyia* is available, even though this genus of sand fly is also implicated in the transmission of leishmaniasis in the New World. In a review of the genetic relationships among species groups within the genus *Lutzomyia* Torgenson *et al.* (2003) stated that: “The structure of the genetic variation among the species analyzed indicated a closer genetic relationship among members of a morphologic group than between members of different groups”. Most of the molecular information found refers to *Lutzomyia longipalpis*, a species common in Brazil, and none to *Lutzomyia verrucarum*. Therefore, any BLAST search done in GeneBank had to rely on the data available for other species of sand flies such as *Lutzomyia longipalpis* when designing primers.

### **Distribution of Phlebotomine Sand Flies**

*Lutzomyia verrucarum* occurs throughout the western half of Peru, from the western slopes of the Andes between 1100 and 2980 m.a.s.l., to the inter-mountain plains between 1200 and 3200 m.a.s.l. As Figure 1 shows, this sand fly’s distribution correlates well with the occurrence of human bartonellosis in most of Peru. In fact, prior to 1997, *Lutzomyia verrucarum* was presumed to be the only vector of human bartonellosis in Peru because it was present in all known foci of the disease (Caceres 1993, Caceres

1997, Tejada et al. 2003). However, discrepancies between the distribution of emerging disease outbreaks and the distribution of *Lu. verrucarum* suggested the existence of secondary vectors in areas where *Lu. verrucarum* is not present (Ellis et al. 1998). This became more evident in 1997 when there was a bartonellosis epidemic in Cusco Department, where *Lu. verrucarum* is absent and the most predominant and only man-biting sand fly species reported is *Lu. peruensis* (Caceres 1997, Ellis et al. 1998).

Risk factors for bartonellosis in endemic areas include association with sand fly bites in and around human dwellings. Because of the poor socio-economic status of the population in the endemic areas, homes from the study area are of adobe construction, generally have few rooms, dirt floors, and no window panes, which allows ready access to sand flies and other biting diptera. These homes are usually just meters away from the occupants' crops, and adjacent to their animals, where sand fly resting and breeding sites may be abundant. Other risk factors include activities, such as napping in the farmland and various agricultural activities after dusk that expose people to sand fly bites. Most transmission appears to occur indoors and in areas surrounding the house (Maguiña, 1998).

Sand fly breeding habitats are basically unknown in Peru and further research is required to locate the development sites of the immature stages. Such information would be useful in developing vector control strategies. Human bartonellosis has a seasonal transmission, with most cases reported in the months of January to June when rainfall is greatest (Pachas 2000). The current study looks at the relationship between the seasonality of the disease in humans and the natural rates of *B. bacilliformis* infections in the sand flies.

## Vector incrimination

Culturing sand flies to determine infection was not done for many years. In 1988, Latorre isolated *Bartonella bacilliformis* from cryopreserved *Lutzomyia*. Because of the limited differential bacteriologic confirmation tools of the time, confirmation of the species was not possible (Latorre et al. 1989). Now with PCR, diagnostic confirmation is highly reliable.

The literature identifies five criteria necessary to establish that a sand fly is a vector of human disease (Killick-Kendrick and Ward 1981). In the case of the transmission of *B. bacilliformis*, these are:

1. The suspected vector sand fly must be anthropophagic and should be present in the area where humans become infected with *Bartonella bacilliformis*.
2. The distribution of the suspected vector should correlate with the distribution of the disease in humans, and sand flies must be abundant to maintain transmission.
3. *Bartonella bacilliformis* should be isolated from wild-caught sand flies and should be indistinguishable from isolates causing disease in humans in the same area.
4. It should be demonstrated that naturally or experimentally infected flies maintain the infection through the complete extrinsic life cycle of the pathogen.
5. Experimental transmission of *Bartonella bacilliformis* by bite or some other means would generally be considered conclusive proof that a sand fly is a vector of a given pathogen.

Due to the lack of a sensitive tool to detect infected sand flies, the last two criteria have not been satisfied, to date, for bartonellosis. The goal of the current research is to develop a sensitive test technique that will permit establishment of the sand fly as the vector for bartonellosis.

## **Prevention and Control**

The first attempt to control sand flies using modern insecticides was carried out by Hertig and Fairchild in January of 1944 using DDT residual spraying in the Rimac Valley in Lima, Peru (Hertig 1948). This was an endemic area of bartonellosis at that time. They tested residual spraying in two adobe huts with a 5% solution of DDT, which protected humans against the bites of sand flies for about a week (Alexander and Marolli 2003). From 1944 to 1993, DDT was successfully used as an insecticide to control insect-borne diseases in Peru, such as malaria. In 1993, the use of DDT was banned, severely limiting the available residual insecticides that could be used for this purpose.

After the discovery of antibiotics, treatments became available using penicillin, chloramphenicol, and tetracycline. These antibiotics were used to treat the symptoms of bartonellosis. These were used to treat patients and helped to control the reservoir that is suspected to be human.

Control measures are usually directed at reducing the population of sand flies with insecticides within a given endemic area and medical attention to cases (MINSA). In Peru, the Ministry of Health uses permethrin as the residual insecticide of choice. Additionally, community-based efforts are encouraged to improve home construction, such as the plastering of adobe walls to eliminate cracks and crevices that might serve as

sand fly breeding sites and patch cracks and crevices in the adobe walls. This disease costs many workdays to the community in an epidemic area and many school hours to the children in an endemic area.

## **Principles of Real-Time PCR**

### **PCR basics**

The polymerase chain reaction is an *in vitro* technique to replicate DNA in a test tube. DNA is unwound and each strand of the target molecule is used as a template to produce new complementary strands. This is repetitive procedure; each repeat is called a cycle. Primers, nucleotides and buffers are required in this process. Machines called thermocyclers are used to change temperatures from hot to cold to denature and anneal the molecules controls this process. New machines, are constantly been developed and increase the sensitivity of the process (McPherson and Moller 2000). There are three basic steps that take place:

1. **Denaturation** at 94-95°C:

During denaturation, the double stranded DNA melts resulting in single-stranded DNA. At this temperature TAQ polymerase is not active.

2. **Annealing** at 54-60°C:

The primers move due to Brownian motion. Ionic bonds are constantly formed and broken between the single-stranded primer and the single-stranded template. When primers sequence matches with its complimentary target more stable bonds will form and a small piece of double-stranded DNA is created. TAQ polymerase recognizes double stranded DNA, attaches and starts copying the template. As more nucleotides are added, the ionic bond is so strong between the template and

the primer that the bond does not disassociate anymore. Most primers will anneal efficiently in 30 sec or less, unless the annealing temperature ( $T_a$ ) is too close to the melting temperature ( $T_m$ ), or the primer sequence is unusually long. The complementary bases to the template are paired to the primer on the 3' end, and the polymerase adds dinucleotides from 5' to 3'.

### 3. **Extension** at 72°C:

To maximize the extension of new DNA, the temperature is increased to 72°C where the polymerase works best. The primers, with a few bases built in, already have a stronger ionic attraction to the template than the forces acting against these attractions. Primers that aligned with mismatched positions are disassociated from the template as the  $T_m$  is increased and will not extend the fragment (Innis and Gelfand 1990).

## **Reaction Analysis**

It is important to confirm that there is a product because not every PCR reaction is successful. Reasons for PCR failure include: the quality of the DNA is poor, bad primer design, inhibitors in the template preparation, or too much starting template. It is possible that a product is made, but it is of a smaller size band than expected from the size of bases. In that case, one of the primers probably fits on a part of the gene closer to the other primer. It is also possible that both primers fit on a totally different gene. To verify that the product obtained is the right size or if more than one band is formed, the PCR amplification product is subjected to gel electrophoresis and ethidium bromide staining or Southern blotting (McPherson and Moller 2000, Innis and Gelfand 1990).

Generally, 25 to 40 cycles are sufficient to produce 100ng -1µg of target sequence DNA from a single copy gene when starting with 50ng of genomic DNA. The number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA (Innis and Gelfand 1990).

### **Real-Time PCR**

In conventional PCR, the amplification product must be detected by gel electrophoresis and ethidium bromide staining. This procedure has several disadvantages: 1) agarose gel analysis has low sensitivity and specificity; 2) Southern blotting is done to confirm the specificity of the product but is time consuming; and 3) The accuracy of these techniques is limited no matter which method is used. When working with the LightCycler instrument, these drawbacks are eliminated, and more precise quantification is possible since this instrument is specially designed for online quantification in real-time.

Real-Time PCR integrates conventional PCR with fluorescent scanning technology, using excited fluorescent dyes that are attached to the specially designed TaqMan® probes or nonspecific dyes such as Sybr Green that will bind to any double stranded DNA. This PCR method uses a system that includes a built-in thermal cycler, an optical source to induce fluorescence, charge-coupled device detector and real-time sequence detection software. The cycle-by-cycle detection of the increase in the PCR product is quantified in real time as the probes are hydrolyzed, *i.e.*, the "reporter dye" fluoresces when the first base of the probe is cleaved away from the remaining probe sequence by the taq polymerase. Once the fluorescently tagged base is separated from the quencher dye, the "quencher" is no longer capable of quenching the reporter dye.

Primers generating small amplification products should be designed for better results, *i.e.*, for greatest sensitivity.

### **Annealing Temperature and Primer Design**

Primer Express® software is a convenient tool to design primers and probes. This design software generates primers and probes optimized for probe hydrolysis reactions, so called TaqMan® Primers & Probe. Primer Express will conveniently select a primer with a G-C content in the 30-80% range. For probe hydrolysis assays, the  $T_m$  should be 68-70°C (approximately 10°C greater than the primer  $T_m$ ) (Innis and Gelfand 1990).

When designing the primers, they should be located as close as possible to the probe sequence without overlapping the probe. Under these circumstances, a more specific amplicon will be generated. From the list of forward primers displayed, the primer selected should have no more than 2 G's and/or C's within the last 5 bases on the 3' end of the primer. If no forward primer matches these criteria, then a primer with 3 G's and/or C's should be selected. Similar criteria are followed for selecting the reverse primers and the probe (Kreuzer et al. 2000).

### **Probe Selection: TaqMan® Probe**

The probe design should insure that the probe has more C's than G's to increase the  $T_m$  and make it more stringent. The probe selected cannot have a G at the 5' end. The Primer Express software allows the option of editing the primer/probe names and adding the reporter/quencher dyes to the probe sequence (Kreuzer et al. 2000).

### **Citrate synthase gene: *gltA***

This gene encodes the sequence for the enzyme citrate synthase. This enzyme is a component of the citric acid cycle, which is important for energy production and biosynthetic precursors. Citrate synthase is found in nearly all cells capable of oxidative metabolism. There are two main types of citrate synthase: a multimeric form found in the Proteobacteria and the dimeric form found in Eukarya. This enzyme has distinct regulatory functions in Proteobacteria and in Eukarya. This gene has been widely been used for phylogenetic analysis for an extensive group bacteria such as *Rickettsiae sp.* and *Bartonella sp.* The sequencing of the citrate synthase in humans has revealed that this gene is located on chromosome 12 (Goldenthal and Marin-Garcia 1998).

A comparison of the *gltA* sequences of the different *Bartonella* species showed similarity between sequences were 83.8 to 93.5%, whereas comparisons of sequences obtained from different strains of the same species revealed that the levels of similarity were greater than 99.8% (Birtles and Raoult 1996). These data reveal that *gltA* sequences are highly conserved among *Bartonella bacilliformis* strains, and thus make this gene a good target for analysis.

Phylogenetic data obtained from various genes including *gltA* allowed the identification of six clusters. *Bartonella bacilliformis* and *Bartonella clarridgeiae* appear to be divergent species. *Bartonella henselae*, *Bartonella koehlerae* and *Bartonella quintana* cluster together, as well as *Bartonella vinsonii* subsp. *vinsonii* and *B. vinsonii* subsp. *berkhoffii* (Houpikian and Raoult. 2001).

## MATERIALS AND METHODS

### Study Site

Sand fly samples were collected from patient and non-patient houses from the villages of Choquechaca, Cullashpampa, Yuracoto and Caraz, all in the Chavin Region of Peru (Figure 2). Sand flies were collected twice a week every other week between May 1999 and December 2000 at each of the four villages.

### Sand Fly Collection

Sand flies were collected with CDC light traps placed inside and outside two different houses per village. Sand flies were attracted to a small battery-operated light in these traps and were swept into a fine-mesh collecting bag by a suction fan. The traps were turned on at 1800 hours and off at 0600 hours; at this time the bags were closed to prevent flies from escaping. The samples were then identified to species, and the females were separated and classified as to whether they had blood meal. Most of the sand flies were *Lutzomyia verrucarum*, but some *Lu. noguchi* were captured. The unfed sand flies were placed in vials containing 80% ethanol, numbered, and labeled with a code, and stored in alcohol until processed for DNA.

Negative control sand flies to *Bartonella bacilliformis*, both fed and unfed, were obtained from colonies reared at Uniformed Services University of the Health Sciences (USUHS) and Walter Reed Army Institute Research (WRAIR). These flies were frozen upon arrival until DNA extraction was conducted.

### Sand Fly DNA Isolation Using Qiagen DNeasy Tissue Kit

Sand flies were removed from alcohol and allowed to air dry for 30 minutes. Then five sand flies were placed in a microfuge tube, and 180µl of ATL buffer and 20µl

of Proteinase K were added from the kit.( Qiagen, Valencia, Calif.). With the help of a sterile grinding pestle, one per pool, sand flies were minced until no recognizable parts remained. Separate pestles were used for each pool to avoid contamination between samples. Samples were incubated at 42°C overnight in a water bath. 200µl of buffer AL was added and vortexed immediately. Tubes were heated in a 70°C heat block for 10 min, and 200µl of 100% EtOH was added and vortexed. The contents of each tube were transferred to a Qiagen spin column in a collection tube. Care was taken to avoid moisture on the rim of the column reduces contamination. The tube was centrifuged at 10,000rpm for 1 minute. The eluent was collected into a clean collection tube and the filtrate discarded. The column was washed with 500µl of Buffer AW1 and centrifuged for 1 min at 10,000rpm. The second wash with 500µl of Buffer AW2, was centrifuged for 3 min at 13,000rpm. The column was centrifuged in a clean tube to collect any remaining buffer. The volume of the elution buffer depends on the number of flies. The pools were eluted with 2 rinses of 50µl of buffer for a final volume of 100µl.

The positive control sand fly samples were spiked with infected red blood cells. These cells were infected using the Trager-Jensen culture method (Trager and Jensen 1976) used for *P. falciparum* and modified for *Bartonella* as follows: ten ml of blood was used. If possible O+, human blood CPD-A, type “O” was ordered, because this anticoagulant will preserve the red blood cells for 35 days (Interstate Blood Bank, Inc., Memphis, TN). The blood was centrifuged to eliminate the serum and the entire buffy coat. The cells were washed with RPMI three times. The red blood cells were then resuspended to 8-12% with RPMI + 10% FBS. For sand fly feeds, FBS was replaced by human serum type “O”. Five ml of this suspension was added to 25cc canted-neck flasks

(Falcon, Franklin Lakes, NJ). The suspension was later inoculated with 1 ml of a *Bartonella bacilliformis* culture. This procedure also can be done using patient blood. The flasks were incubated in a candle jar at 28°C for three days. A control flask without inoculation of a *B. bacilliformis* strain was maintained each time. The culture was monitored by taking using a drop of the sediment and preparing a thin smear and a thick smear every other day after fifth day of culture. Slides were stained with Giemsa (Sigma) to detect bacillary forms. Sometimes the cultures would be predominantly coccoid, and more fresh blood was added to induce transformation to bacilli. Once they were in the bacillary form, the culture was ready to spike or feed sand flies. Sand flies were spiked with infected blood as follows: 0.2ul for 1 sand fly, 0.4ul for 2 sand flies, 0.6ul for 3 sand flies, 0.8ul for 4 sand flies and 1ul for 5 sand flies. The DNA extraction protocol previously described using Qiagen DNeasy Tissue Kit for sand flies was followed after spiking.

#### **DNA extraction of *Bartonella* sp. strains with Qiagen DNA kit**

Bacterial strains of *Bartonella bacilliformis* were grown in Columbia Broth Agar (BBL, Cockeysville, MD) with 10% sheep blood and overlaid with 5 ml of RPMI + 10% Fetal Bovine Serum were added, and these cultures were then incubated at 28°C. Strains isolated from previous studies were kept at -70°C and were thawed and cultured in the medium described above. The flasks were monitored every other day to check for contamination. Strains usually grew after 8-15 days, and a suspension of the liquid phase was used for the extraction. The DNA's from the strains of *Bartonella henselae*, *Bartonella vinsonii*, *Bartonella quintana* and *Bartonella elizabethae* were supplied by

AFIP with a known concentration of 1ng/ul each, and these were used for specificity testing.

Citrated blood samples and strains grown in biphasic medium were extracted by using the DNeasy (Qiagen, Valencia, Calif.) tissue kit following the manufacturer's protocol and eluted in to final 200µl volume. The DNA of strains was quantified as references to use later in the standardization of the assay using the Beckman DU 640 spectrophotometer.

### **Conventional PCR**

All the conventional PCR studies were done at USUHS. The initial experiments using PCR assays in sand flies used primers for *Bartonella henselae* to detect the citrate synthase gene (Norman et al. 1995). This test used the PCR Master kit (Roche Applied Science, Indianapolis, IN) according to the following protocol: (Mix for 1 tube times the number of samples to be tested) 25µl of PCR Master Mix, 15µl of PCR water, 2.5µl of 781p at a 2 µM stock for a final concentration of 0.1 µM, 2.5µl of 1137n at a 2 µM stock for a final concentration of 0.1 µM and 5µl of template (DNA). The forward primer used was the 781P, GGGGACCAGCTCATGGTGG, and the reverse primer was the 1137N, AATGCAAAAAGAACAGTAAACA (Norman et al. 1995).

A MJ Research thermocycler was used following the *Bartonella henselae* citrate synthase (BHCS) protocol. Each reaction involved an initial 2-min denaturation at 95°C prior to thermal cycling. Each cycle consisted of a 30" denaturation at 95°C, a 1-min annealing at 50°C, and a 1-min extension at 72°C. Amplification was concluded after a total of 35 cycles and a final 10-min extension at 72°C. Reactions were subsequently maintained at 4°C until analyzed by gel electrophoresis.

## **Electrophoresis for DNA detection**

Gels were prepared with TBE buffer molecular biology grade (Quality Biological Inc.) and Agarose-1000 (GibcoBRL) at a concentration of 2.0% and boiled for 1 minute. The sample was cooled to 40-50°C before adding 5ul of ethidium bromide solution, 10 mg/ml (GibcoBRL), then poured into a tray. Wells were loaded with 8µl of sample (DNA) with 2µl glycerol gel loading dye 5X (AMRESCO) to a final 1:5 dilution. After all the samples were loaded, the gels were run at 80V for 1-2 hours. Gels were photographed using a Gel Doc 100 (BIO RAD) that includes a digital camera and saved as TIFF files for further analysis.

## **Real Time PCR using the LightCycler thermocycler**

### **Primer design**

Several sets of primers were design with Primer3 Output a program available at [http://flypush.imgen.bcm.tmc.edu/primer/primer3\\_www.cgi](http://flypush.imgen.bcm.tmc.edu/primer/primer3_www.cgi). The complete sequence of the *gltA* gene of *Bartonella bacilliformis* available in GeneBank, accession number Z70021, was used to generate a real time probe hydrolysis assay. The annealing temperature was 60°C, and the primer sequence was around 20bp long. These primers were made at AFIP using the ABI394 DNA synthesizer. The primers were desalted and purified.

After trying out the primers previously described without good results, new primers were designed using Primer Express program and the sequence information of GeneBank. The accession numbers of the following *Bartonella bacilliformis* analyzed were as follows: AY114114, AY114113, AY114117, AY114111, AF478357, AF478356,

AY114116, AY114118, AY114115, AY114112, BBU28076, AY114119, and Z70021 were analyzed. According to the alignment results, AY114111 was selected as the reference strain to be used against the other species. The sequences of a variety of *Bartonella* species were analyzed to find a variable area of the gene among species. The accession numbers of the sequences for the citrate synthase gene aligned for the analysis are listed in Table 1 by accession number. The BCM Search Launcher available online was used for the sequence alignment results of both searches. The web page is:

[http:// searchlauncher.bcm.tmc.edu/multi-align/multi-align.html](http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

The portion of the variable sequence was then introduced to Primer Express software (Perkin-Elmer Corp/Applied Biosystems) TaqMan Primer & Probe Design option and three initial set of primers were selected. Desalted grade primers were ordered from Invitrogen, Life Technologies. Upon arrival primers were reconstituted with PCR-grade water to 1ml vials stock of 100 $\mu$ M each and stored until use.

### **Basic Real-time reaction set up**

A major concern with PCR amplification is contamination, which can be caused by PCR products amplified in other tubes in the same assay or caused by amplicons from previous PCR assays. Every reaction mix was prepared inside a hood after dressing in clean scrubs. The sets of primers were tested initially with Sybr Green to determine the efficiency of the primers. Each amplification mix contained final concentrations of 200  $\mu$ M of each deoxynucleoside triphosphate (dNTPs) (Idaho Technology, Salt Lake, Utah), 2 U of iTaq DNA polymerase (BIORAD, Hercules, CA), 250nM concentrations of each primer, PCR water, 1x IT Buffer and 1x IT enzyme diluent (Idaho Technology, Salt Lake, Utah). Table 2 shows a set up for a single reaction. Sybr Green dye (Roche

Applied Science, Indianapolis, IN) was used in the initial screenings to a final concentration of 1:30,000, and later replaced by the probe. The total master mix reaction volume of 18  $\mu$ l was dispensed into LightCycler Capillaries (Roche Applied Science, Indianapolis, IN). Two microliters of the purified DNA template was added out of the hood with a different set of pipettes for a final 20- $\mu$ l PCR volume. After one-pulse centrifugation to mix and send the reaction mix into the distal end of each cuvette, the tubes were placed in the LightCycler carousel carefully in order not to break the fragile capillaries. The program parameters used initially was the 2-step + Melt. The cycling conditions were as follows: 45 cycles with each cycle consisting of 0 seconds of denaturation at 95°C, and 20 seconds of annealing at 60°C. It had an additional melt step of 10 minutes. Reaction products were subsequently maintained at 4°C until analyzed by agarose gel electrophoresis if necessary.

### **Optimization of primer concentrations**

A master mix without primers was first prepared. A panel of 25 tubes with different concentrations of each reverse and forward primer was tested, starting from dilutions of primers to 1 $\mu$ M, 2.5 $\mu$ M, 5 $\mu$ M, 7.5 $\mu$ M and 10 $\mu$ M of each forward and reverse primer, and combining them in the master mix (Table 3). Each primer combination was tested in duplicate. Because of the capacity of the LightCycler is 32 capillaries per run, the total optimization was done in two runs. The highest fluorescence and the lowest CT value (crossing the threshold) were used to determine the selection of the optimal dilution. The CT value is the number of cycles needed for the fluorescence signal to be detected. This number correlates inversely to the amount of DNA in the template.

### **Optimization of probe concentration**

Once the primer concentration was optimized, the probe was tested to find its optimal concentration. Taqman® TAMRA™ Probes are labeled with 6FAM™ at the 5' end, and TAMRA™ at the 3' end. Probes were purchased from Applied Biosystems, Foster City, CA.

Five stock concentrations of probe were prepared: 0.5μM, 1μM, 2μM, 3μM and 4μM for a final concentration of 50nM, 100nM, 200nM, 300nM and 400nM, respectively. The probe concentrations were tested with the optimal primer concentrations to determine the maximum amount of signal generated by the primer-probe combination. Once the probe was optimized, it replaced the Sybr Green for more specific results. Sybr intercalates in the DNA so there is more dye to generate signal in the double stranded molecules. The probe only reacts with the probe site and has to be hydrolyzed in the reaction to generate a signal. Consequently probe LODs are usually not as sensitive as the Sybr assays.

### **Limit of detection quantification.**

The limit of detection (LOD) for each assay was evaluated with samples of purified DNA quantitated using a spectrophotometer. DNA was extracted from 200μl of each sample and eluted in 200μl of extraction buffer. Subsequently, 2μl of the template DNA was used per PCR reaction. Freshly prepared ten-fold serial dilutions of the strain HOSP800-29 at concentrations of 1ng/5μl, 100pg/5μl, 10pg/5μl, 1pg/5μl, 100fg/5μl, 10fg/5μl and 1fg/5μl. These were tested for the limit of detection. Frozen dilutions of DNA are not recommended for the 30/30 studies (testing a positive control at the lowest

detectable concentration, 30 capillaries in the same test with a positive result on the 30 samples) because the expected results may vary. The 2-step + Melt protocol was followed using the same reagents from the probe optimization step.

The initial limit of detection was determined by the lowest dilution at which both replicates were positive. To confirm this result, a 30/30 study was performed. Thirty replicates of the dilution were tested with one known positive of 100pg/5µl and a non-template control. The LOD should be done with the lowest concentration that gives positives in the initial evaluation of the template concentrations. An LOD of 100 pg seems very high since positive are usually in the femtogram range. The test was then repeated with a higher dilution if the initial dilution was not 100 percent positive. Therefore, the LOD is the lowest concentration of sample usable, which results in 100% positive after thirty repeats. (Be aware that this is not the lowest concentration that can be detected but the concentration that is detected 100% of the time.)

### **Specificity**

*Bartonella bacilliformis* specificity was subsequently conducted with a battery of templates from closely related and more distantly related organisms. All the primers and probes were screened for specificity with template DNA from *B. henselae*, *B. quintana*, *B. vinsonii* and *B. elizabethae*. Twelve strains of *Bartonella bacilliformis* were used as positive controls: HOSP800-29, Sandi, HOSP800-09, PAT360, CUS006, CUS005, PAT300, HOSP800-72, HOSP800-76, 10502 (CUSCO), VER0075, and CUS80804.

### **Analysis of the amplicons with Agilent**

The products were tested most of the time with the regular gel electrophoresis procedure described previously, but some of the products were analyzed with Agilent 2100 Bioanalyzer. Agilent uses a DNA 500 LabChip Kit, (Agilent Technologies, Palo Alto, CA). This is also called “lab on a chip”. It is a semi-automated capillary electrophoresis device that can size the amplicons in less than two minutes. It is a very sensitive tool that gives quick results with several options of analysis.

### **Sequencing of the amplicons**

The conventional PCR products were purified using the Qiaquick Spin kit following the protocol provided by the manufacturer (Qiagen Inc, Valencia, CA). PCR products were sequenced in both directions using terminator cycle sequencing with the Reaction Premix kit (BIC, USUHS, MD).

A reaction mix was prepared as follows: 4μl of reaction mix, 2μl of primer, 4μl of water and 10μl of purified DNA for a final volume of 20μl. This was done for each primer and cycled together for 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 min and a final step at 4°C for 30 minutes in the thermocycler. The products were then delivered to the USUHS Molecular Biology Core Laboratory where they were processed in the Applied Biosystems Prism 3100 Genetic Analyzer.

### **Statistical analysis**

The sensitivity of each procedure for the detection of *Bartonella bacilliformis* was determined by dividing the number of true positive results by the number of positive

strains of *Bartonella bacilliformis*. The specificities of the assay were calculated by dividing the number of true negative results by the number of negative controls.

## RESULTS

The initial aim of this study was to detect sand flies infected *in vitro* to incriminate *Lutzomyia verrucarrum* as the vector of Carrion's disease. Our initial results, using conventional PCR were variable. Therefore, we searched for a more sensitive tool that would be able to detect infected individual flies.

### Cultures

Cryopreserved strains from previous studies were thawed and placed into standard biphasic media used to grow *Bartonella* and incubated at 28°C. Canted neck flasks were used because they sustain the humidity in the flasks. Plates were not used because they dry out after a couple of weeks and are easily contaminated. *Bartonella bacilliformis* often takes several weeks to months to appear as colonies on solid media. Several passes may be required until a strain is adapted to laboratory conditions. Once a culture has grown in a biphasic media it remains viable up to 8 weeks, after, which the organisms are subcultured to maintain the strain. Strains were frozen for future use. After 8-15 days, the cultures were ready for inoculation using the Trager-Jensen method (Malaria culture method). Fresh cultures were preferred as the inoculum for these studies; human red blood cells in CPDA-1 were used for all the media. These conditions mimic the inoculum of a natural infection. Cultures were tested for density and morphology by making thick and thin blood smears and staining the slides with Giemsa. If any flask showed a change of color or hemolysis, it would be discarded, because *Bartonella* should

not hemolyze cells *in vitro* or acidify the media. Thus, a control flask of washed cells RBC is incubated along with the infected culture flasks to avoid an excess of manipulation of the flasks. It is not possible to add any antibiotics to the media because *Bartonella* is very sensitive to most of them. Consequently, a septic technique is extremely important in successfully growing *Bartonella*. Usually the red cell cultures were ready to be checked from the third day of inoculation up to the seventh day. Five-day cultures generally would have multiple bacilli forms (Figure 3). Slides that had more than two clusters per one field were considered as good positive cultures. *In vitro* *Bartonella* grow mostly in the media (extraerythrocytic), and usually in clusters, thus, making individual quantification very difficult. That is why clusters were counted, instead of individual bacteria, to assess the density of the culture. After the organisms were in the bacillary form, they were ready to be fed or to spike the sand fly preparations. Cultures were then centrifuged at 3000 rpm for 10 min and reconstituted to a 35-40 percent of red cells by volume. A single flask containing a total of 6 ml would give a final working solution of 1.5-2.0 ml of cells mixed with extracellular bacteria. Strain HOSP800-09 constantly yielded better growth with this method.

### **Conventional PCR results**

A battery of *in vitro* infected sand flies was tested following the PCR protocol BHCS. While the controls worked well, very few of the experimental samples gave positive results. At most only 10 percent or sometimes fewer of the samples were positive, when tested. We considered the possibility that sand flies did not have a complete meal, and thus another strategy was chosen.

In this second approach, sand flies were spiked with known amounts of blood,, 2µl per fly, and these too gave poor results. Single flies gave less than 50% positive results, but the results improved when the number of flies in a pool was increased. Pools of 3, 4 and 5 flies spiked with 0.6, 0.8 and 1µl of blood respectively, infected from a preparation showing a multiple clusters of bacteria on a slide, gave reliable results (Table 4). All analyses were done using gel electrophoresis.

This procedure was repeated several times with the same results; DNA extractions were repeated to ensure that the DNA extraction procedure was not interfering with the results. DNA was measured to determine the amount in the template, but it was not possible to distinguish between the DNA from the bacteria and the DNA from the sand fly. Extractions of 0.2, 0.4, 0.6 and 0.8µl had similar readings making it impossible to extrapolate results. This outcome produced enhanced efforts for arrangements to work at AFIP where the technology and equipment was available.

### **Real Time PCR with the LightCycler**

Several tests were done using the Norman *et al.* primers. In a first run, reagents and primers used in previous USUHS studies for conventional PCR were tested against the same primers with reagents used at AFIP using a 3-step PCR cycle of 30', 60', 60'. Three positive controls were used: Sandi, HOSP800-09 and HOSP800-29. This last strain was at concentration of 2.74ng/µl. The other concentrations of DNA preparations were undetermined because they came from boiled strains and DNA concentration was not possible, but the DNA concentration was considered to be much higher. The analysis showed that the reagents used at AFIP were more sensitive, *i.e.*, smaller amounts of DNA were detected in the test, but with low fluorescence (Figure 4).

A 3-step PCR program with cycles of 30', 30', 30' was analyzed with the same combination of reagents as above. The analysis showed no amplification with USUHS reagents, and the amplification with AFIP reagents was not consistent.

A third program was tried using the following cycles 5', 20', 20', and the results as the remained inconsistent. These results confirmed that the AFIP reagents were capable of amplification using short cycling times, and that a better primer set needed to be designed that would work with Real Time PCR. Amplicons of 100bp or less are desired, and an annealing temperature of 60<sup>0</sup>C was recommended for primer annealing.

### **First Primer Design**

Two sets of primers were designed with Primer3 Output, a computer program. The complete sequence of the *gltA* gene of *Bartonella bacilliformis* available in GeneBank, accession number Z70021, of 910bp, was used for this design, and an annealing temperature of 60<sup>0</sup>C was selected to give optimal specificity for annealing of the primers. The result gave one forward primer and two reverse:

GLTA-F1 CTT TAT GGT GAG TTG CCA AAC A

GTLA-R1 GCA GAC ATA GCA CCA AGA CAA G

GTLA-R2 CAG ACA TAG CAC CAA GAC AAG C

The program 2-step + melt (previously described in Material and Methods) was used for the run with working stocks of primers of 2.5μM. There was no amplification detected during real-time PCR, and analysis was done using Agilent 2100 Bioanalyzer to confirm our negative results. Even though this method is sensitive, no trace of amplification was observed.

### **Primer design with Primer Express**

Various genes have been used in PCR assays for *Bartonella bacilliformis*. Previous work on sand flies indicated the citrate synthase gene was a reliable gene to identify *Bartonella* because it does not cross react with *Lutzomyias* genes (Gordon et al. 2000). Also, there is more information on that gene in *Bartonella*. An analysis of *Bartonella bacilliformis* AY114114, AY114113, AY114117, AY114111, AF478357, AF478356, AY114116, AY114118, AY114115, AY114112, BBU28076, AY114119, and Z70021 led to the selection of the AY114111 strain for our studies. This sequence was compared to other *Bartonella* species to find the most variable section of sequence between *B. bacilliformis* and other *Bartonella* species. For the first three primers sets, a portion of the total sequence from the strain AY114111 was selected and introduced to the Primer Express software.

BBF1 TCG TCT TAT GGG CTT CGG C

BBR1 ACG TCT TTT GGA CGG TAC TT

BBF2 CCC ACT TTT TGA TAT AGC GAT GG

BBR2 AAT TGT TTT TCG AGA TAG GAT TAC AGCT

BBF3 CAT TCA AGA TAA CCC ACT

BBR3 CGT GTA GCG AGA CTT ACT CTT

### **Screening of the primers**

An initial run was done using Sybr green (stock diluted 1: 3000), working stocks of 2.5µM primers and undiluted template DNA. Duplicate capillaries of each DNA from three strains of *Bartonella* were used for all the screenings: Sandi, HOSP800-09 and HOSP800-29. PCR results were variable according to the primer set used.

### **Optimization of primer concentration**

Three sets of primer combinations were tested, BBF1-BBR1, BBF2-BBR2 and BBF3-BBR3, using Sybr Green to determine the efficiency of the experimental system. The first set, BBF1-BBR1, gave the best results and was then optimized. It gave the highest fluorescence and the lowest CT value when compared to the other primer combinations. Tube 12 showed the best primer combination. The primer concentration for BBF1 and BBR1 was 250nM for the forward and 500nM for the reverse (Figure 5).

The second set of primers had low fluorescence and very late CT, but the results were consistent. The primer concentration for BBF2 and BBR2 selected was 750nM for the forward and 500nM for the reverse (Figure 6).

Tubes 7 and 12 had very similar results for the third set of primers, but the results were not consistent. The primer concentration for BBF3 and BBR3 was 250nM for the forward and 500nM for the reverse (Figure 7).

### **Optimization of probe concentration**

For the first sets of primers, 2 probes were designed: Bart-1 for the BBF1-BBR1 set and BART-MGB for the BBF3-BR3 set.

Bart-1 6FAM ACA GAG TCT ACA AAA ATT ATG ATC CAC GTG CAA  
AGA TTT TAMRA

BART-MGB 6FAM TTG ATA TAG CGA TGG AGC TT MGBNFQ

Using the optimal primer dilution, each probe was screened with an initial 1µM stock to check on the efficiency. BART-1 gave amplification products for the positive controls and was subsequently tested with five stock concentrations of probe: 0.5µM, 1µM, 2µM, 3µM and 4µM for a final concentration of 50nM, 100nM, 200nM, 300nM

and 400nM, respectively. The optimal dilution for BART-1 was 2μM, but the fluorescence was low and the amplification curve was unusual (Figure 8). The results for BART-MGB showed a less unusual amplification curve, but there were no amplification products for some of the positive controls (Figure 9). Therefore, no probe optimization was done for BART-MGB.

The ABI7700 was used to improve the amplification curve using BBF1-BBR1 with probe BART-1 at 2μM. The amplification results looked better but had less fluorescence than with the LightCycler.

### **Specificity**

Once the amplification curve with probes was improved with the ABI7700, specificity was tested using Sybr Green. Pools of 10 negative *Lutzomyia verrucarum* were processed for DNA. Surprisingly nearly all the negative control specimens from the sand flies were positive, and the NTC control was negative (Figure 10). The results suggest either the template or some of the reagents were contaminated. Because the negative control was negative, a new batch of sand flies was extracted using a new DNeasy tissue kit to eliminate the possibility of contaminated reagents. The new batch of flies included more species (*Lutzomyia longipalpis*, *Phlebotomus papatasi* and *Anopheles sp.*). These species were pooled in groups of 5 to match the number in field sand fly samples. This new batch gave fewer positives. Negative fly pools included the following:

#### *Lutzomyia verrucarum*

25 pools of 10 flies each (first batch)

4 pools of 10 flies each, 1 of 5 flies (second batch)

*Lutzomyia longipalpis*

2 pools of 10 flies each, 1 of 5 flies (second batch)

*Phlebotomus papatasi*

2 pools of 10 flies each, 1 of 5 flies (second batch)

*Anopheles sp.*

2 pools malaria negative

Decreasing the number of cycles was not a possibility due to the low CTs of the positive pools. The most reasonable explanation was the primers were cross-reacting with *Lutzomyia* genes. The best solution to the problem was to design new primers to avoid cross-reactions.

### **Last Set of Primers**

The design for these primers was different; the citrate synthase gene was still used as the target and the primer sets were targeted throughout the gene. Primer Express was used, and seven new sets of primers were ordered (Invitrogen, Life Technologies).

Primer set 1: 5'-3' BB154 GCG GCA TAC AAT GGT GCA T

BB224R CCATAG GAT GAG AAT CGC GAC

Primer set 2: 5'-3' BB175 ACA ATT TGC ACG CTT CTT CCA

BB249R GCA CCA AGA CAA GCG ACC A

Primer set 3: 5'-3' BB511 ATG AAC AAA ATG CAT CTA CGT CAA C

BB586R GCT GCA ATA CAT GCA TAC GGA T

Primer set 4: 5'-3' BB584 ACA GTA CGC CTT GCA GGT TCA

BB609R GTC CCC AAA GGC ATG CAA C

Primer set 5: 5'-3' BB151 GCG GCA TAC AAT GGT GCA T

BB216R GGA TGA GAA TCG CGA C

Primer set 6: 5'-3' BB198 TTC GTC GCG ATT CTC ATC CT

BB260R AAA ATG CAG ACA TAG CAC CAA GAC

Primer set 7: 5'-3' BB531 ACA GTA CGC CTT GCA GGT TCA

BB697R GGC ATG CAA CAC CTG CTG

### **Screening of the second batch of primers**

These screenings also used Sybr green to a final concentration of 1:30,000, an arbitrary working stock of 2.5µM of primers, controls and undiluted template DNA. The positive controls were *Bartonella* strains: Sandi and HOSP800-29. A negative sand fly pool NEGPOOL1 was also included in every set to determine if the primers were amplifying with sand flies. The four first sets of primers were screened in one test (Figure 11). After the test, all the amplicons were refrigerated for subsequent gel electrophoresis analysis.

Primer set 1 gave positive signals with all the samples including the NTC; the negative pool gave a CT of approximately 28; this result looked positive. The melting curve gave some hope of success; the melting temperature of positive samples was 83°C and the negative pool had a melting point of 87°C. They were far enough apart to be different bands (Figure 12).

Primer set 2 gave positive results in all the samples including the NTC, and the negative pool gave a CT of 25. The melting curve showed the positive samples had a melting temperature of 84°C, and the negative pool had a melting point of 86°C; this result represents only a small difference in temperature but still enough to produce different bands (Figure 13).

Primer set 3 was negative for the NTC's and positive for all the samples. The negative pool gave a CT of approximately 32. The melting curve showed the positive samples had a melting temperature of 82°C, and the negative pool had a melting point of 84°C. This primer was a strong candidate for our study (Figure 14).

Primer set 4 was positive with all the samples including the NTC; the negative pool gave a CT of 28. The melting curve showed that the positive samples had a melting temperature of 84°C, and the negative pool had a melting point of about 82.5°C, which is only a small temperature difference (Figure 15).

The last three sets were tested together using the same controls and samples as the other primers (Figure 16). The fluorescence was not as high, and the following results were obtained:

Primer set 5 was positive for all the samples including the NTC with a low CT; the negative pool gave a CT of 26. The melting curve showed the positive samples had a melting temperature of 83°C, and the negative pool had a melting point of 86°C (Figure 17).

Primer set 6 was positive for all the samples including the NTC; the negative pool gave a CT of 25. The melting curve showed the positive samples had a melting temperature of 82°C, and the negative pool had a double peak with melting points of 79°C and 85°C (Figure 18).

Primer set 7 was positive to all the samples including the NTC; the negative pool gave a CT of 28. The melting curve showed the positive samples had a melting temperature of 83°C, and the negative pool had a double peak with melting points of 79°C and 87°C (Figure 19).

These results suggested to us that the primers were amplifying a target in the NTC's. In fact, this is probably a primer dimer of some sort, although it seems to have a high melting point.

Gel electrophoresis of the amplification products of primer sets 3, 5, 6 and 7 showed the primers had non-targets in the NTC, *i.e.*, a primer dimer and a specific amplicon were observed in the samples containing target DNA. Primer set 3 had no band in the non-template control but had a band of the expected size of  $\approx 75$ bp. Primer set 6 had weak bands for the positives (Figure 20). To be certain that a good candidate primer was not overlooked, a new test with sets 1, 2, 4 and the BBF1-BBR1 was run with the same controls and a different negative sand fly pool, NEGPOOL2. The results were very similar to the results found in the first test. The sand fly pool was positive with most of primer sets according to their melting curves. The gel electrophoresis analysis of primer sets 1, 2, 4 and BBF1-BBR1 showed that all the primers had multiple bands for the negative sand fly pool, but none with the same size as the positive control (Figure 21). Primer set 2 had the same band pattern for the NTC and the negative sand fly pool, but the expected band for a positive was absent. These results were encouraging, but with a probe it would be possible to get more specific. In summary, using this approach we had generated two candidate sets to optimize and test with a respective probe.

### **Optimization of Primer Set 3 BB511-BB586R**

The optimization protocol with Sybr Green described before was used for our selected primers. The result for primer set 3 showed that tube 8 with 500nM for the forward and 250nM for the reverse had the lowest CT and the highest fluorescence.

Therefore it was selected as the optimal primer dilution (Figure 22). Some of the non-template controls gave some amplification with a different melting point.

### **Optimization of Primer Set 2 BB175-BB249R**

The optimization protocol was done with Sybr Green. The optimal primer concentration for this assay was 250nM for the forward primer and 500nM for the reverse primer (Figure 23). All the non-template controls had some amplification product with a high melting point, but the  $T_m$  was different than that expected for the specific amplicon.

Field samples were extracted and tested using Set2 primers and Sybr Green while we were waiting for new probes. Most all the samples generated amplicons, and thus it was necessary to analyze results using the melting curves. Four samples were true negatives with Sybr Green; nine samples gave questionable results. A gel was run and multiple bands were visible, including a band the size expected for a positive test (Figure 24).

### **New Probes BB2-203, BB3-543**

For the selected primers, 2 probes were designed: probe BB2-203 for primer set2  
probe BB3-543 for set3:

BB2-203 6FAMCGT CGC GAT TCT CAT CCT ATG GCT GTTTAMRA,

BB3-543 6FAMTAC GCC TTG CAG GTT CAT CAG GAG CTAMRA.

Upon arrival, the new probes were tested with concentrations of 0.5 $\mu$ M, 1 $\mu$ M and 2 $\mu$ M with control DNA at a10pg concentration and with the four questionable samples from the Sybr Green assays. Set2 with its respective probe gave encouraging results. The NTC control was negative. The control strain gave a negative reaction with the 0.5 $\mu$ M

probe concentration but was positive with the 1 $\mu$ M and 2 $\mu$ M probe concentration. The questionable samples all gave negative results.

Primer set3 was tested at the same time, and the control strain gave negative results with the 2 $\mu$ M probe concentration but positive results with the 0.5 $\mu$ M and 1 $\mu$ M probe concentration. The NTC control was negative. The questionable samples gave 1 positive, 1 negative and two samples were positive again. An agarose gel was run with these amplicons, and single bands were observed. The positive sample with probe 3 had the same size band as the positive controls (Figure 25).

### **Optimization of Probe BB2-203**

Three stock concentrations of probe were prepared: 1 $\mu$ M, 2 $\mu$ M and 3 $\mu$ M for a final concentration of 100nM, 200nM and 300nM, respectively. These were tested against tenfold serial dilutions of *Bartonella bacilliformis* strain, HOSP800-29 with concentration of 100pg/5 $\mu$ l, 10pg/5 $\mu$ l, 1pg/5 $\mu$ l and 100fg/5 $\mu$ l. This test showed working stocks of 2 $\mu$ M that gave a higher fluorescence and lower CT's (Figure 26).

### **Optimization of Probe BB3-543**

Due to the results in the previous run with probe BB3-543, only two stocks of probe were prepared 1 $\mu$ M and 2 $\mu$ M. These were tested against tenfold serial dilutions of strain of HOSP800-29 with concentrations of 1ng/5 $\mu$ l, 100pg/5 $\mu$ l, 10pg/5 $\mu$ l, 1pg/5 $\mu$ l, 100fg/5 $\mu$ l, 10fg/5 $\mu$ l and 1fg/5 $\mu$ l. This test showed that working stocks of 1 $\mu$ M gave a highest fluorescence and lower CT's (Figure 27).

### **Limit of detection quantification.**

The LOD for this assay was determined by tenfold serial dilutions of strain HOSP800-29 (1ng, 100pg, 10pg, 1pg, 100fg, 10fg and 1fg). The 2-step + Melt protocol was used with amplicons forming in all dilutions except the last one (Figure 28).

The initial limit of detection was determined as 10fg, and a 30/30 study was performed. The 30 replicates of the dilution were tested with one known positive and a negative control. This test gave positive results on 19/30 samples, therefore the next higher dilution was tested. The second 30/30 study was done at a concentration of 100fg concentration of DNA. This time, 100% of the capillaries were positive. Although the LOD could have been refined, for practical purposes, the LOD was established at 100fg when using probe BB2-203 (Figure 29).

### **Sensitivity test for Probe BB2-203**

A total of 12 strains of *Bartonella bacilliformis* were tested with this primer-probe combination, and all of them gave a positive signal in every test. Strains from Cusco and Caraz, Peru were used (Table 4).

### **Specificity test for Probe BB2-203**

Along with the positive strains, other species of *Bartonella* were tested with this probe. *Bartonella henselae*, *Bartonella vinsonii*, *Bartonella quintana* and *Bartonella elizabethae* were supplied by AFIP, and only *Bartonella vinsonii* gave a weak positive signal (Figure 30). Two negative pools were included, one gave a positive result with the expected band and the other was negative.

Gel electrophoresis was performed showing a 75bp band in all the amplicons. The negative pools gave multiple bands, and the “positive” pool gave a 75bp band as well (Figure 31).

### **Troubleshooting**

The negative pools were still generating probe positive results, and these were from flies reared in laboratory conditions. They had never been outside the laboratory. There are a couple of possible explanations: either there was a cross-reacting gene in the sand flies (there isn't any gene sequence information for sand flies in GeneBank), or the sand flies were actually infected transovarially or transtadially. This last argument could be true for the simple reason that one of researchers, an entomologist working with sand flies, had visited endemic areas of bartonellosis. There was the possibility he was an asymptomatic patient. He fed the colonies by exposing his arms and allowing the flies to feed. There was a last but more remote possibility that transovarian transmission of *Bartonella* from the parental flies had occurred. In an attempt to determine the presence or absence of *Bartonella* being transferred by the flies, a new batch of unfed sand flies was extracted. During the extraction process, most of the flies appeared to contain blood.

In addition, a 5ml blood sample from P.L., was drawn for culture, PCR and serology. Two flasks containing biphasic media were inoculated with 1ml of blood each and incubated at 28°C for two months. The cultures were checked regularly every week, but no growth was found after the complete period of incubation. Two vials of DNA were extracted from the blood sample and were tested with primers BB175 -BB249R and probe BB2-203 with 2step + melt program. Three control strains were included along with DNA extracted from different volumes of low infected red blood cells. All the

samples generated the 75bp Amplicon in this test, including the vials from P.L. blood. The results of the test are shown in Figure 32.

To insure that the primers did not cross react with human DNA, another 14 human samples were tested, including people in close contact with sand flies from the colonies or who had been in endemic areas of Peru. Cultures were included in 6 of the 14 samples; the remaining 8 samples were from patients from Brazil who were negative for malaria (no cases of bartonellosis has ever been reported in Brazil). Only one of the samples gave low fluorescence. The test was repeated at a 1:10 dilution and was negative. The amplicon from the P.L sample was sequenced at AFIP and USUHS. The final count of human DNA positive for *Bartonella* by PCR was 1 out of 15 (6.67%).

A new batch of unfed sand flies were pooled and extracted for DNA. These flies were tested along with guinea pig blood and mouse blood. This time one pool out of 35 was positive. This pool was from the *L. verrucarum* colony, which has anthropophilic preferences and were fed by P.L. It is possible that a fly that appeared to be unfed was included in the pools extracted for DNA. Apparently sand fly genes were not cross-reacting with the test (Figure 33). Archived field samples were extracted for the test.

### **Sequence results**

The Amplicon sequence results from real time PCR were inconclusive; apparently there was mixed Amplicon present. There were 66 nucleotides available to blast. The information from the Blast search was: *Mus musculus* chromosome 9, clone...38 bits, *Homo sapiens* BAC clone RP11-89K2... 38 bits, *Botrytis cinerea* strain ... 38 bits, *Caenorhabditis elegans* cosmid C18A3...38 bits, *Mus musculus* BAC clone RP23-3C16...36 bits, *Homo sapiens* PAC clone RP5-953B5...36 bits, *Homo sapiens*

chromosome 17, clone...36 bits, *Homo sapiens* chromosome 17, clone...36 bits, *Homo sapiens* chromosome 8, clone...36 bits, *Homo sapiens* 3 BAC RP11-789F5...36 bits, *Homo sapiens* chromosome 8, clone... 36 bits, *Homo sapiens* chromosome 8 clone ...36 bits, *Homo sapiens* chromosome 8, clone...36 bits, *Homo sapiens* chromosome 8, clone... 36 bits, *Homo sapiens* IRSp53 gene for in...36 bits, Human DNA sequence from clone ... 36 bits, *Homo sapiens* genomic DNA, chromosome...36 bits. This information was not conclusive, and a new sequencing effort had to be done.

To improve the amount of information derived a conventional PCR was tried with samples from P. L. and J. G. The post amplification agarose gel analysis of both samples showed bands of 379bp, the same size as the positive control (Figure 34). These products were purified and prepared for sequence and sent again to AFIP and USUHS.

The second results of sequences gave the following blast search:

Sequences producing significant alignments:	(bits)	Value
gi 18921333 gb AC098648.2  <i>Homo sapiens</i> chromosome 3 clone ...	464	e-128
gi 18093008 gb AC092051.2  <i>Homo sapiens</i> chromosome 3 clone ...	464	e-128
gi 32423805 gb AY254308.1  <i>Bartonella chomelii</i> citrate synt...	46	0.042
gi 845661 gb L38987.1 BAOGLTA <i>Bartonella henselae</i> citrate s...	46	0.042
gi 4929446 gb AF148484.1 AF148484 <i>Bartonella</i> sp. pb18975nm ...	44	0.17
gi 31282333 gb AY125039.1  <i>Zaglossus bruijni</i> recombination ...	42	0.66
gi 15824430 gb AF303971.1  <i>Tachyglossus aculeatus</i> recombina...	42	0.66
gi 39226738 gb AC096689.5  <i>Oryza sativa</i> chromosome 3 BAC cl...	40	2.6
gi 16413128 emb AL596166.1  <i>Listeria innocua</i> Clip11262 comp...	40	2.6
gi 37990751 dbj AK121128.1  <i>Oryza sativa</i> (japonica cultivar...	40	2.6
gi 24196184 gb AE011410.1  <i>Leptospira interrogans</i> serovar l...	40	2.6
gi 23477778 gb AC134929.1  <i>Oryza sativa</i> (japonica cultivar-...	40	2.6
gi 32990294 dbj AK105085.1  <i>Oryza sativa</i> (japonica cultivar...	40	2.6
gi 32977592 dbj AK067574.1  <i>Oryza sativa</i> (japonica cultivar...	40	2.6

Apparently the cross-reaction was to a gene in the human chromosome 3 and was not positive *Bartonella*. Human citrate synthase gene is located in chromosome 12. Information on the normal gene expression on chromosome 3 was searched to find an explanation why only some human DNA amplified with our primers. There was no information on the gene expression of that portion of the gene. Therefore, the field samples were processed.

### **Field samples**

A few of the pools from field samples showed strong positive reactions. Many samples appearing weakly positive were negative (Figure 35). The inability to definitively determine positive from negative pools was a limitation in this analysis. Many of the questionable samples were repeated with the result of more effort, time and consumption of reagents. Still the results were not clear enough.

One of the advantages of the LightCycler is the ability to monitor PCR in real time. Many of the true positives showed amplification before the 36th or 37th cycles. Analysis of the data, including the LOD results, suggested that limiting the number of cycles in the PCR assay would prevent nonspecific amplification. The 2-Step and Melt protocol were modified to 40 cycles instead of the 45 original cycles.

The new 2Step and Melt protocol gave better results and less questionable amplifications. All the questionable samples were repeated, and this time it was evident which samples were positive. Due to technical problems, not all the samples extracted were processed; *i.e.*, the laptop attached to the LightCycler crashed and has not been repaired. A total of 472 pools were tested with the primers BB175-BB249 and the probe

BB2-203. Positive controls were used in every test, with a positive result on every one of them. We conclude that the test has a sensitivity of 100% and specificity 91.11%. Of the 472 pools tested, 13 were positive (2.75%). House A had the more pools tested than any other house; of 343 pools, 8 were positive (2.33%), House G had 39 pools tested with 3 positives, and House I had 29 pools tested and 2 of these pools were positive (Table 5). Of the 13 positive pools, 5 were collected outside the house (38.46%) and 8 were collected inside the house (61.54%). Samples from some months were not available from the archive, so there are some gaps in the distribution of the samples in the seasonal variation analysis. Table 6 show the results of all the samples tested.

## DISCUSSION

*Bartonella bacilliformis* is small and can be easily confused with any bacterium contaminating sand flies. Therefore, dissection of sand flies to detect these bacteria is not a possibility. Studies by Hertig in the 1940's mentions that bacteria which had been observed in the sand fly's proboscis using an optical microscope indicated that the fly was positive for *Bartonella bacilliformis*. Since then, many new and more specific and sensible techniques have been developed to characterize such findings.

Studies to determine the vector of bartonellosis began at the Uniformed Services University in 1995. At the beginning, conventional PCR techniques using the citrate synthase gene were used to detect infected sand flies. However, this method lacked the desired sensitivity and specificity for field studies because the amount of DNA target in the sample was very small. The purpose of the present study was to investigate the effectiveness of real time PCR assay carried out on naturally and experimentally infected sand flies. Real time PCR has been key in the development of new diagnostic techniques

for fastidious bacteria. This tool has been rapidly improved. Real time measurements have increased the possibility of studying fastidious bacteria because of the test's high sensitivity and faster processing.

The developed technique allows detecting minuscule amounts of *Bartonella bacilliformis* DNA that is the expected concentrations to be found in sand flies. This diagnostic tool will enable scientists to incriminate sand flies as the vectors of bartonellosis without doubt in further studies, but it has some limitations. Cross-reaction with human DNA is a possibility when using the citrate synthase gene as marker. The current primer sets detected the citrate synthase gene of *Bartonella* and also a portion of human chromosome three. This chromosome was detected with both Real Time PCR and with conventional PCR. In our study 6.67% of human DNA was positive for the *gltA* gene when using our primers. The human DNA positive with our assay, along with a sample that gave a questionable result which was repeated with a negative final result, were tested with conventional PCR. Both samples were positive with conventional PCR. It is possible that samples that tested negative with Real Time PCR could be positive in conventional PCR tests. This shows that our assay is more specific than conventional PCR. The blast result of the sequence of the conventional PCR amplicon showed a perfect alignment with 222bp of the human chromosome three. Information on the normal gene expression of that portion of the chromosome was not possible through the Gene Atlas web page and similar pages of universities that worked in the human genome project. A possible explanation to why only some human DNA was amplified with citrate synthase primers would be that the gene amplified in the chromosome 3 expresses some normal characteristic true in both subjects and is not common in the rest of human DNA

tested. This explanation is based on the fact that that both cross-reactive samples were Caucasians while the negative samples came mostly from Hispanic individuals. This cross-reaction with human DNA is an important consideration when using citrate synthase gene as a target for *Bartonella* in human samples. Sequencing of the amplicons is highly recommended to avoid false positives.

Therefore, negative pools of sand flies should be obtained from newly emerged sand flies to avoid the risk of human blood interference or cross-reaction. Specificity could be improved if we ensure that only unfed flies are used in the negative DNA pool.

The amplification products of the positive samples of this study were not sequenced because the primers and probe covered most of the region amplified, leaving only seven nucleotides available for a mismatch (Figure 36).

A tool to confirm our positive samples for this study besides sequencing the amplicons, could be to design primers for a different gene that does not amplify human DNA. The riboflavin (*ribC*) gene may be an alternate site to target because no cross-reaction occurs with human samples (Bereswill et al. 1999, Johnson et al. 2003, Zeater et al. 2003).

When evaluating the specificity of the primers and probes, the panel of bacterial DNA from other *Bartonella* species showed that there was a cross reaction with *Bartonella vinsonii*. The reason that the *B. vinsonii* DNA was recognized was that there is only a single nucleotide difference exists in the target sequences between it and *Bartonella bacilliformis*. The use of a labeled probe to differentiate the strains from each other was not as useful as expected when differentiating *Bartonella bacilliformis* from *Bartonella vinsonii*.

One of the goals of this study that we were not able to accomplish was to analyze the cycles of *Bartonella* infection in sand flies and their seasonal variations. Samples from some months were not available. Apparently samples were misplaced or relocated without communication from the laboratory technician. A new group of samples should be collected and studied for confirmation of our data (Figure 37).

Field sample pools were tested in a blinded study. Vials were numbered which did not reflect the source of the pools of the flies. The analysis of the positive field pools gave important and interesting information. All the positive samples came from patient houses, and no positives were obtained from non-patient houses. The infection rate of sand flies observed in this study was 2.75% of the tested specimens without blood in their guts. The incidence of infected flies (positive PCR test) may vary due to the low number of pools processed from some patient and non-patient houses. It should be noted that most of the positive samples came from the same patient house where more sand fly pools had been obtained. If we take into account only the information from house A, a lower percentage of infection in the sand flies, 2.33% would have been calculated (Figure 38). When comparing the results of the three houses with the positive PCR sand fly pools, it was noted that most positive samples were collected during the month of March 2000 (Figure 39). Collections from consecutive years should be tested to confirm this information. Other than this single observation, seasonal variations were not evident. The information of location of the collected samples did not give a significant difference because the positive samples collected outside the house were 38.46% of the total positive pools. While, positive pools collected inside the house were 61.54% of the total positive results of our study. There were more samples collected inside than outside as

shown in Table 6. These numbers were not comparable because more pools were obtained from outside collections than from inside.

A study to determine seasonal variability should be designed using the incident rate reported in our study and the appropriate number of sand flies collected each month of the year. These data would provide a good analysis of seasonal variation with a high probability of detecting positives when they are present. Increasing the number of houses per village would also improve our understanding of sand fly infection and transmission of *Bartonella*. It would confirm our data and provide a better epidemiologic understanding of the relationship of infected sand flies to positive patients. Our data shows sand flies can be infected with *Bartonella* after feeding on infected blood. Our work provides methods and direction for future studies to determine if sand flies only get infected after feeding on infected humans. We still do not understand if sand flies transmit the infection or only serve as incidental hosts for *Bartonella*.

Mechanical transmission of disease pathogens occurs when a vector transports organisms, such as bacteria that cause dysentery, on its feet, body hairs and other body surfaces to the host. The vector does not play an essential role in the biological development or proliferation of this pathogen. Direct inoculation of infected red blood cells (RBC's) from the gut of a sand fly would also be considered mechanical transmission if there was no multiplication or development of the pathogen within the vector's body. This type of transmission is more or less incidental.

The infection rate of this study is slightly higher than in the study done with conventional PCR by Gordon *et al.* (2000). This indicates that our numbers are in the expected rates. Other studies done in sand flies to detect other diseases have had a

variety of results; *Wolbachia* was found in sand flies (60 to 81%) with PCR (Benlarbi and Ready, 2003). A study using Real-time PCR to detect infection of *Leishmania* in sand flies found an average of 2.9% infection rate of this disease (Svobodova et al, 2003). Infection rates of 11.1% for *Bartonella henselae* have been found in fleas (Rolain et al. 2003).

The relatively small scale of this study dictates that any conclusions drawn from the infection rates of *Bartonella bacilliformis* in sand flies and the epidemiological data available from the positives should be considered with care.

## CONCLUSIONS

Our PCR analysis exhibited a sensitivity of 100% and a specificity of 91.11%. These results are related to the short time needed to reach the annealing temperature, which reduces the nonspecific annealing of primers, the  $T_m$  of the primers and the variation in  $T_m$  between specific Amplicon sequences and nonspecific Amplicon sequences. Positive real-time DNA assays should be tested with conventional PCR, followed by sequencing of the Amplicon until we can find DNA sequence specific for *Bartonella bacilliformis*.

The two-step PCR procedure in the LightCycler thermal cycler is a versatile technique allowing both real-time analyses with Sybr-green and specific probe hydrolysis assays. The required amount of DNA is small, 1-5  $\mu$ l. DNA preparations from sand flies, even those extracted after a blood meal, did not contain inhibitory substances for PCR. This suggests our DNA extraction protocol was good and establishes a baseline for future comparisons of extraction protocols employing sand flies.

Although neither *Bartonella bacilliformis* or any of the other *Bartonella* species have been fully sequenced, we can estimate the size of the genome as 5MB. If this is correct, then our assay was detecting approximately 2-20 copies of the target sequence in the pools. This method has the necessary sensitivity and specificity. It is a valuable tool that will enable us to determine the true infection rates of *Bartonella bacilliformis* in wild-caught sand flies from endemic areas of Peru. In addition, PCR allows screening of large numbers of samples synchronously, as is required for epidemiological studies.

Application of PCR in well designed and carefully controlled experiments will provide data defining the role of the sand flies as vectors of *Bartonella bacilliformis*.

## APPENDICES

Table 1. *Bartonella bacilliformis* strains used from GeneBank.

Accession number	specie	Length of bases
AF440274	<i>Bartonella sp.</i>	338
AF440273,	<i>Bartonella sp.</i>	338
BECSF9251	<i>Bartonella elizabethae</i>	965
AY114111	<i>Bartonella bacilliformis</i>	331
AF214557	<i>Bartonella henselae</i> <i>subsp. arupensis</i>	980
AF143445	<i>Bartonella vinsonii</i> <i>subsp. berkhoffii</i>	929
BAOGLTA	<i>Bartonella henselae</i>	
BHE439406	<i>Bartonella henselae</i>	927
BQCSFULLR		
AY035823	<i>Bartonella quinatana</i>	483
BVU28075	<i>Bartonella vinsonii</i> <i>subsp. berkhoffii</i>	338
BQU28073	<i>Bartonella quintana</i>	338

Table 2. 1X PCR reaction set up

Master Mix	1X reaction
PCR water	6µl
10x IT buffer	2µl
10x IT DNTP	2µl
Forward primer 2.5µM	2µl
Reverse primer 2.5µM	2µl
Probe or (Sybr Green 1:3000)	2µl
10x IT enzyme diluent	1.84µl
Platinum taq 5 units/µl	0.16µl

Table 3. Primer optimization check board.

4µl of each primer dilution was added to each tube.

#1: 100F, 100R	#2: 250F, 100R	#3: 500F, 100R	#4: 750F, 100R	#5: 1000F, 100R
#6: 100F, 250R	#7: 250F, 250R	#8: 500F, 250R	#9: 750F, 250R	#10: 1000F, 250R
#11: 100F, 500R	#12: 250F, 500R	#13: 500F, 500R	#14: 750F, 500R	#15: 1000F, 500R
#16: 100F, 750R	#17: 250F, 750R	#18: 500F, 750R	#19: 750F, 750R	#20: 1000F, 750R
#21: 100F, 1000R	#22: 250F, 1000R	#23: 500F, 1000R	#24: 750F, 1000R	#25: 1000F, 1000R

Table 4. Conventional PCR results.

# of FLIES	VOLUME SPIKED	PCR RESULT
1	0.2µl	NEGATIVE
2	0.4µl	NEGATIVE
3	0.6µl	POSITIVE
4	0.8µl	POSITIVE
5	1.0µl	POSITIVE
10	2.0µl	POSITIVE
0	0.2µl	NEGATIVE
0	1.0µl	POSITIVE

Table 5. *Bartonella bacilliformis* strains used as controls.

STRAIN	LOCALITY	TYPE OF SAMPLE
HOSP800-29	CARAZ	ACUTE
SANDI	CARAZ	ACUTE
HOSP800-09	CARAZ	ACUTE
PAT360	CARAZ	CHRONIC
CUS006	CUSCO	ACUTE
CUS005	CUSCO	ACUTE
PAT300	CARAZ	CHRONIC
HOSP800-72	CARAZ	ACUTE
HOSP800-76	CARAZ	ACUTE
10502	CUSCO	ASYMPTOMATIC
VER0075	CARAZ	CHRONIC

Table 6. Distribution of results by houses.

House letter	Type of house/place	Number of pools tested	Positives
House A	Patient/Choquechaca	343	8 (2.33%)
House B	Non-patient/Choquechaca	26	0 (0%)
House C	Patient/Caraz City	10	0 (0%)
House D	Non-patient/Caraz City	2	0 (0%)
House G	Patient/Cullashpampa	39	3 (7.69%)
House H	Non-patient/Cullashpampa	0	0 (0%)
House I	Patient/Yuracoto	29	2 (6.89%)
House J	Non-patient/Yuracoto	15	0 (0%)
House K	Non-patient/Cullashpampa	8	0 (0%)

Table 7. Field sample results.

Date of collection	Collection #	House Letter	Inside house	Outside house	Total <i>Lutzomyia verrucarum</i>	# not bloodfed L. ver.	# of pools	PCR
19-May-99	CAR 0240	G	1		6	5	1	POS
25-May-99	CAR 0251	A		1	81	58	11	NEG
25-May-99	CAR 0250	A	1		109	17	3	NEG
25-May-99	CAR 0254	C	1		16	6	1	NEG
26-May-99	CAR 0264	C	1		29	11	2	NEG
01-Jun-99	CAR 0272	I	1		48	8	1	NEG
03-Jun-99	CAR 0283	I		1	7	5	1	NEG
03-Jun-99	CAR 0282	I	1		27	7	1	NEG
08-Jun-99	CAR 0287	A		1	82	42	8	1,2,4-8 NEG 3POS
08-Jun-99	CAR 0286	A	1		132	12	2	NEG
08-Jun-99	CAR 0288	B	1		8	5	1	NEG
08-Jun-99	CAR 0290	C	1		18	8	1	NEG
09-Jun-99	CAR 0294	A		1	9	5	1	NEG
16-Jun-99	CAR 0303	G		1	5	5	1	NEG
23-Jun-99	CAR 0329	A		1	28	19	3	NEG

30-Jun-99	CAR 0338	G	1		23	20	4	NEG
30-Jun-99	CAR 0339	G		1	7	5	1	POS
07-Jul-99	CAR 0368	B	1		5	5	1	NEG
31-Aug-99	CAR 0501	A		1	27	25	5	NEG
15-Sep-99	CAR 0541	A		1	80	70	14	NEG
21-Sep-99	CAR 0552	I	1		38	11	2	1 POS, 2NEG
22-Sep-99	CAR 0556	G	1		21	11	2	NEG
22-Sep-99	CAR 0558	I	1		35	9	1	NEG
22-Sep-99	CAR 0561	J		1	6	5	1	NEG
28-Sep-99	CAR 0563	A		1	70	64	12	NEG
28-Sep-99	CAR 0562	A	1		18	16	3	NEG
28-Sep-99	CAR 0564	B	1		6	5	1	NEG
28-Sep-99	CAR 0565	B		1	31	17	3	NEG
28-Sep-99	CAR 0566	C	1		11	8	1	NEG
29-Sep-99	CAR 0571	A		1	7	6	1	NEG
06-Oct-99	CAR 0582	I	1		28	8	1	NEG
25-Jan-00	CAR 0815	G		1	8	6	1	NEG
26-Jan-00	CAR 0822	I	1		38	13	2	NEG
09-Feb-00	CAR 0844	I	1		72	20	4	NEG
10-Feb-00	CAR 0852	I	1		71	11	2	NEG
15-Feb-00	CAR 0856	A	1		24	17	3	NEG
15-Feb-00	CAR 0857	A		1	55	41	8	NEG
15-Feb-00	CAR 0858	B	1		10	7	1	NEG
16-Feb-00	CAR 0864	A	1		6	5	1	NEG
23-Feb-00	CAR 0880	I	1		17	5	1	NEG
29-Feb-00	CAR 0885	A		1	42	36	7	NEG
29-Feb-00	CAR 0884	A	1		47	36	7	NEG
29-Feb-00	CAR 0889	C		1	7	5	1	NEG
01-Mar-00	CAR 0892	A	1		10	8	1	NEG
01-Mar-00	CAR 0893	A		1	6	5	1	NEG
08-Mar-00	CAR 0908	I	1		18	5	1	POS
14-Mar-00	CAR 0913	A		1	24	20	4	NEG
14-Mar-00	CAR 0912	A	1		8	5	1	NEG
15-Mar-00	CAR 0921	A		1	45	34	6	NEG
15-Mar-00	CAR 0920	A	1		27	19	3	POS
15-Mar-00	CAR 0922	B	1		21	18	3	NEG
21-Mar-00	CAR 0928	G	1		15	14	2	NEG
21-Mar-00	CAR 0930	I	1		19	9	1	NEG
21-Mar-00	CAR 0932	J	1		9	5	1	RPT
22-Mar-00	CAR 0934	G	1		8	6	1	POS
22-Mar-00	CAR 0938	J	1		8	6	1	NEG
30-Mar-00	CAR 0948	A	1		11	5	1	NEG
04-Apr-00	CAR 0956	G	1		14	12	2	NEG
04-Apr-00	CAR 0958	I	1		28	7	1	NEG
04-Apr-00	CAR 0961	J		1	7	7	1	NEG
11-Apr-00	CAR 0968	A	1		35	20	4	NEG
11-Apr-00	CAR 0969	A		1	44	21	4	NEG

11-Apr-00	CAR 0971	B		1	8	7	1	NEG
11-Apr-00	CAR 0970	B	1		16	11	2	NEG
12-Apr-00	CAR 0977	A		1	73	49	9	1-5,7-9 6=POS
12-Apr-00	CAR 0976	A	1		38	20	4	NEG
12-Apr-00	CAR 0978	B	1		9	7	1	NEG
12-Apr-00	CAR 0979	B		1	11	8	1	NEG
18-Apr-00	CAR 0986	I	1		25	8	1	NEG
19-Apr-00	CAR 0990	G	1		57	36	7	NEG
19-Apr-00	CAR 0993	I		1	12	9	1	NEG
19-Apr-00	CAR 0992	I	1		20	10	2	NEG
19-Apr-00	CAR 0994	J	1		17	12	2	NEG
25-Apr-00	CAR 0997	A		1	47	42	8	NEG
25-Apr-00	CAR 0996	A	1		72	60	12	NEG
25-Apr-00	CAR 0998	B	1		13	11	2	NEG
26-Apr-00	CAR 1004	A	1		47	38	7	3? NEG
26-Apr-00	CAR 1005	A		1	34	24	4	NEG
26-Apr-00	CAR 1009	C		1	8	5	1	NEG
26-Apr-00	CAR 1008	C	1		11	6	1	NEG
03-May-00	CAR 1014	I	1		59	15	3	NEG
04-May-00	CAR 1020	I	1		41	9	1	NEG
09-May-00	CAR 1024	A	1		30	18	3	NEG
09-May-00	CAR 1025	A		1	114	80	16	NEG
10-May-00	CAR 1033	A		1	79	54	10	NEG
10-May-00	CAR 1032	A	1		62	42	8	NEG
10-May-00	CAR 1034	B	1		6	5	1	NEG
10-May-00	CAR 1036	C	1		15	9	1	NEG
17-May-00	CAR 1042	I	1		31	5	1	NEG
18-May-00	CAR 1046	G	1		9	8	1	NEG
25-May-00	CAR 1061	A		1	43	26	5	NEG
01-Jun-00	CAR 1068	G	1		39	28	5	NEG
01-Jun-00	CAR 1072	J	1		5	5	1	NEG
02-Jun-00	CAR 1074	G	1		32	19	3	NEG
02-Jun-00	CAR 1078	J	1		8	5	1	NEG
06-Jun-00	CAR 1080	A	1		16	8	1	NEG
06-Jun-00	CAR 1081	A		1	33	15	3	NEG
07-Jun-00	CAR 1089	A		1	51	37	7	NEG
12-Jun-00	CAR 1096	G	1		11	10	2	NEG
12-Jun-00	CAR 1100	J	1		8	5	1	NEG
04-Jul-00	CAR 1137	A		1	58	49	9	NEG
05-Jul-00	CAR 1145	A		1	90	74	14	NEG
05-Jul-00	CAR 1146	B	1		8	6	1	NEG
11-Jul-00	CAR 1152	G	1		8	6	1	NEG
11-Jul-00	CAR 1155	K		1	5	5	1	NEG
18-Jul-00	CAR 1168	A	1		23	16	3	NEG
25-Jul-00	CAR 1187	K		1	6	5	1	NEG
26-Jul-00	CAR 1195	K		1	8	5	1	NEG
02-Aug-00	CAR 1201	A		1	56	41	8	NEG 5,8=POS
02-Aug-00	CAR 1200	A	1		39	31	6	NEG, 5=POS

03-Aug-00	CAR 1209	A		1	42	35	7	NEG
10-Aug-00	CAR 1228	I	1		16	5	1	NEG
15-Aug-00	CAR 1233	A		1	14	8	1	NEG
15-Aug-00	CAR 1232	A	1		37	24	4	NEG
16-Aug-00	CAR 1241	A		1	39	28	5	NEG
16-Aug-00	CAR 1240	A	1		56	41	8	NEG
23-Aug-00	CAR 1254	J	1		8	7	1	NEG
24-Aug-00	CAR 1256	G	1		11	10	2	NEG
29-Aug-00	CAR 1265	A		1	18	15	3	NEG
29-Aug-00	CAR 1266	B	1		8	5	1	NEG
30-Aug-00	CAR 1273	A		1	44	36	7	NEG
30-Aug-00	CAR 1272	A	1		38	21	4	NEG
06-Sep-00	CAR 1291	K		1	6	6	1	NEG
12-Sep-00	CAR 1295	A	1		47	26	5	NEG
13-Sep-00	CAR 1303	A	1		36	17	3	NEG
19-Sep-00	CAR 1311	G	1		9	6	1	NEG
19-Sep-00	CAR 1317	J	1		11	11	2	NEG
19-Sep-00	CAR 1318	J		1	10	10	2	NEG
19-Sep-00	CAR 1313	K	1		9	9	1	NEG
19-Sep-00	CAR 1314	K		1	12	9	1	NEG
20-Sep-00	CAR 1326	J		1	11	5	1	NEG
20-Sep-00	CAR 1322	K		1	14	11	2	NEG
26-Sep-00	CAR 1328	A		1	36	23	4	NEG
26-Sep-00	CAR 1330	B		1	8	7	1	NEG
27-Sep-00	CAR 1335	A	1		84	58	11	NEG
20-Dec-00	CAR 1529	A		1	170	150	30	NEG
20-Dec-00	CAR 1531	B		1	17	16	3	NEG
20-Dec-00	CAR 1530	B	1		12	10	2	NEG
20-Dec-00	CAR 1533	C		1	8	6	1	NEG
20-Dec-00	CAR 1535	D		1	14	14	2	NEG
26-Dec-00	CAR 1536	G	1		17	12	2	NEG
TOTAL			85	56			472	

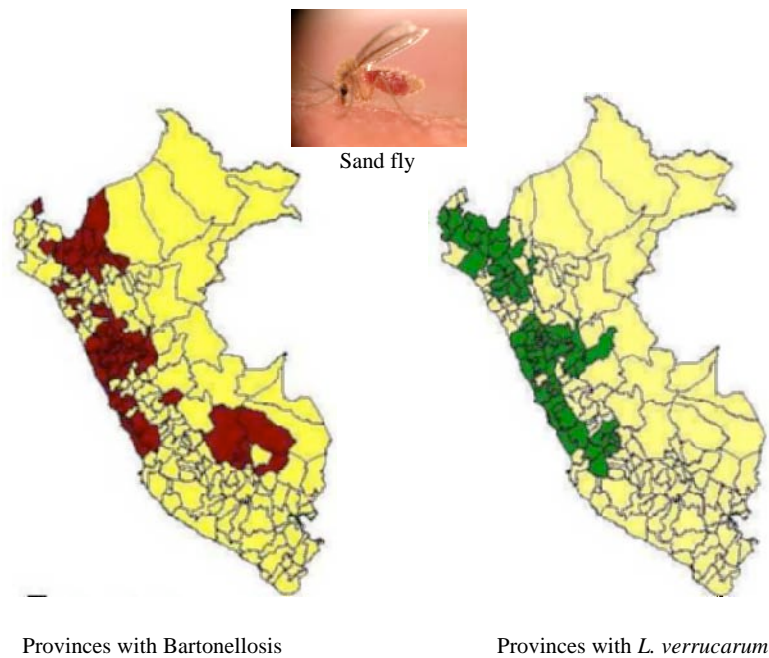


Figure 1. Correlation of Bartonellosis cases with *Lutzomyia verrucarum* distribution

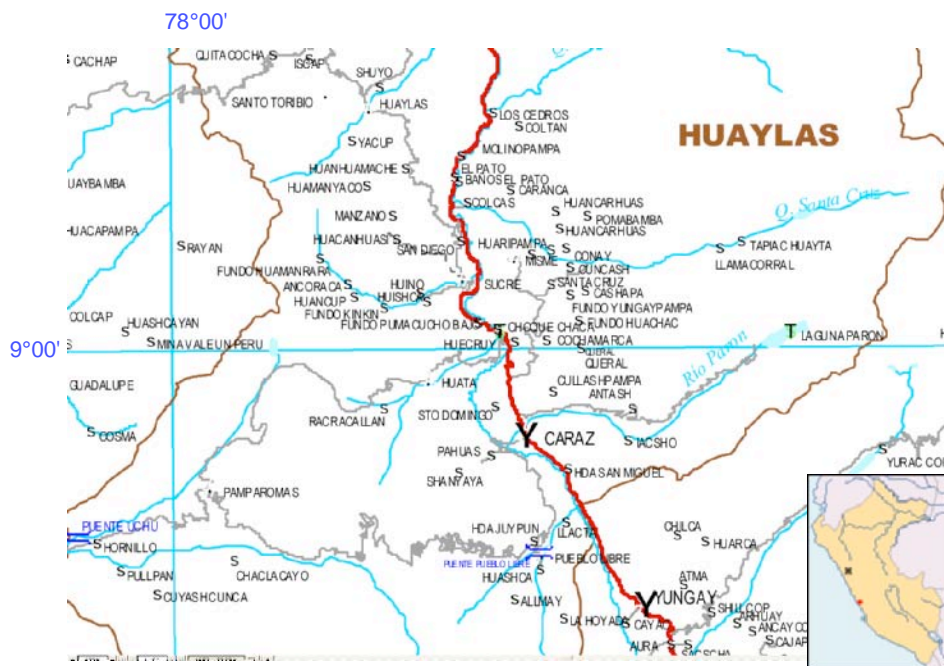


Figure 2. Map of study area showing towns of Choquechaca, Cullashpampa, Yuracoto and Caraz. Red line represents the main road to the area.

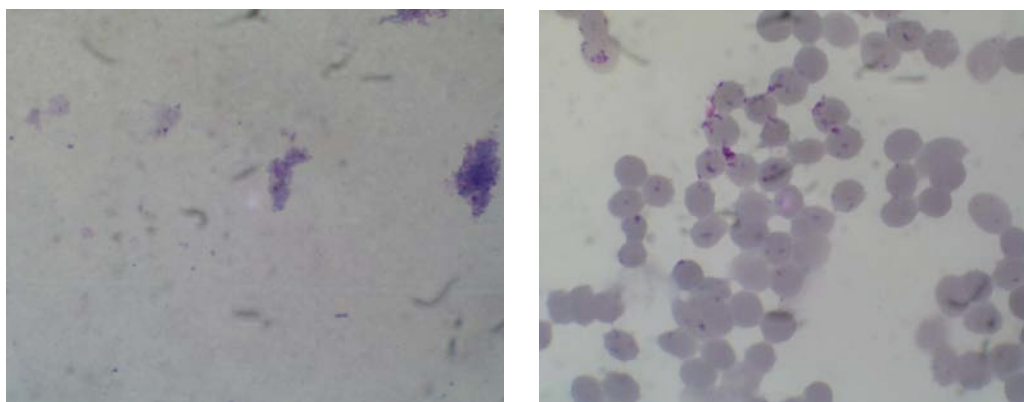


Figure 3. The photo to the left is a thick smear of 5-day *Bartonella bacilliformis* culture. The photo at the right side shows a thin smear of the same culture. Slides are stained with Giemsa.

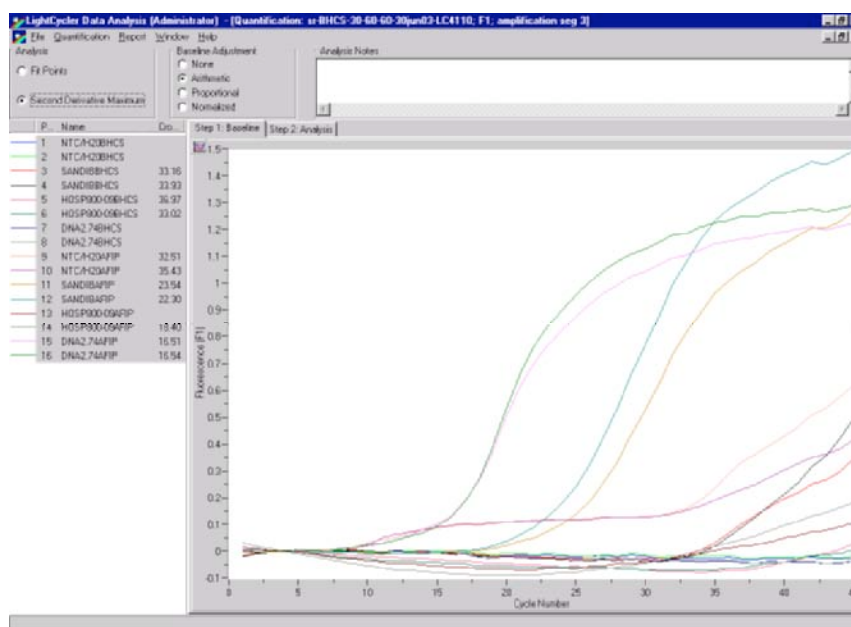


Figure 4. Test with USUHS reagents and primers (samples 1 - 8) compared to AFIP reagents and USUHS primers (samples 9 - 16) in the LightCycler with a 3 step PCR: 30-60-60. The graphic shows that AFIP reagents worked better.

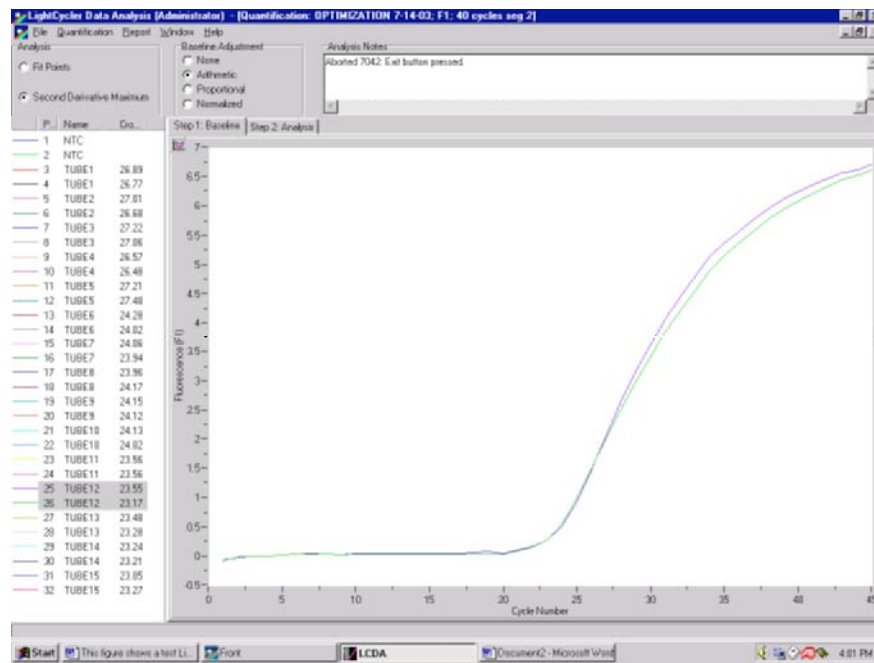


Figure 5. Results of optimization experiment testing primers BBF1-BBR1. Tube 12 is best combination, concentration 250 forward, 500 reverse.

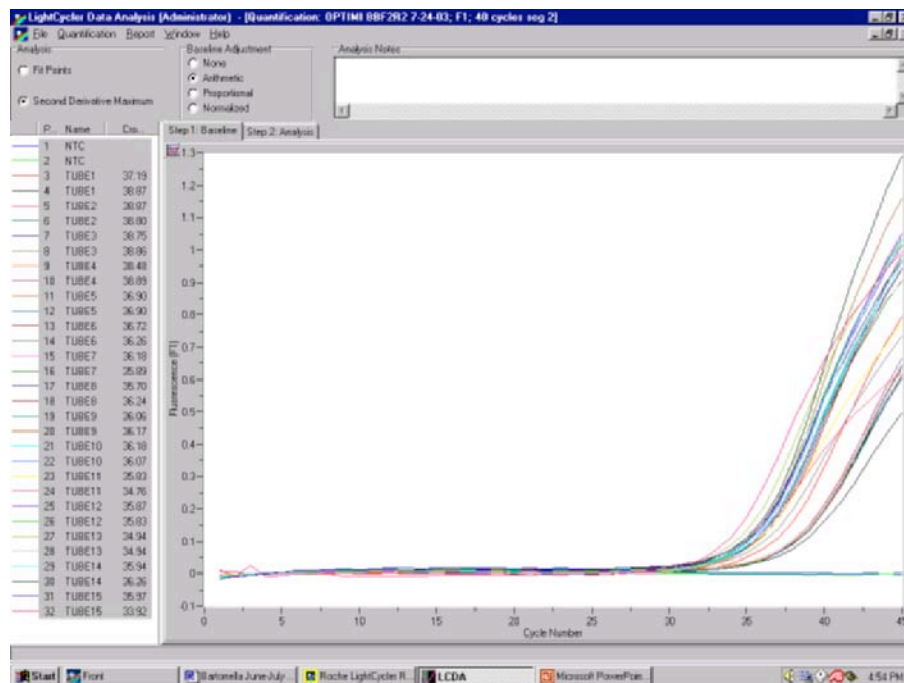
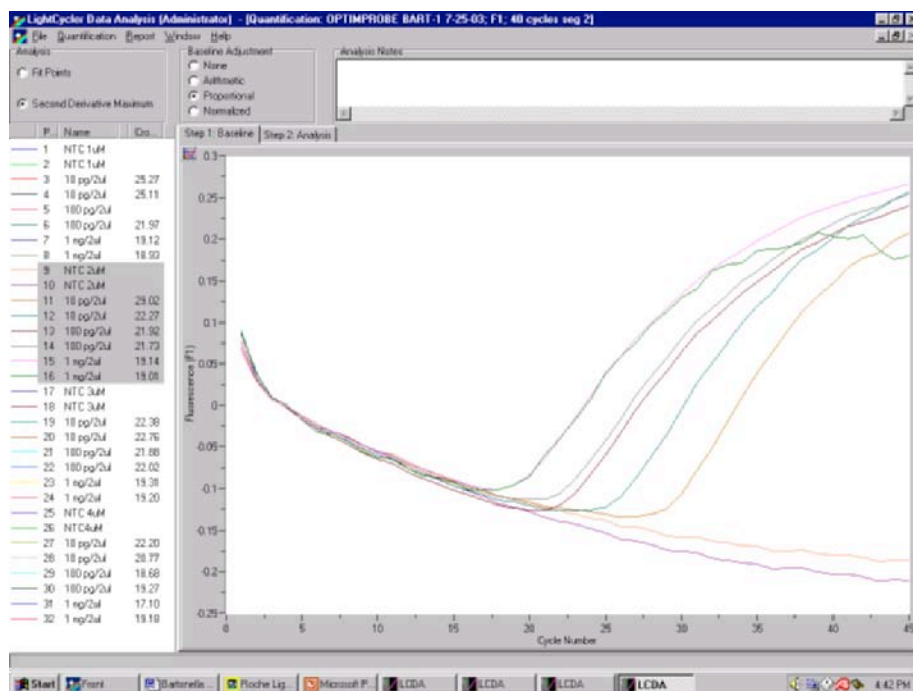
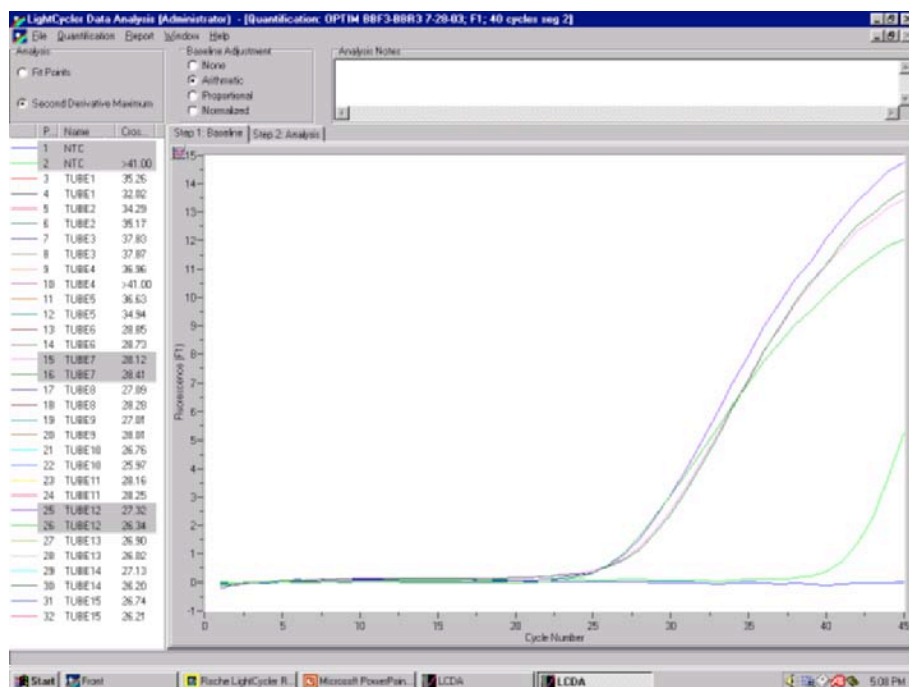


Figure 6. Optimization of primer concentration for primers BBF2 and BBR2.



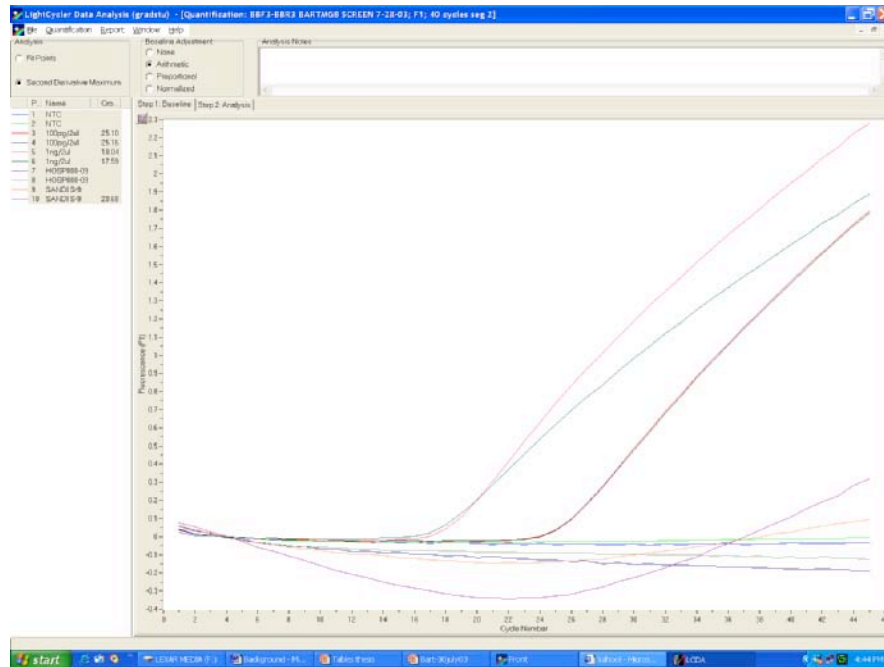


Figure 9. BART-MGB screening showed no amplification for some of the positives controls.

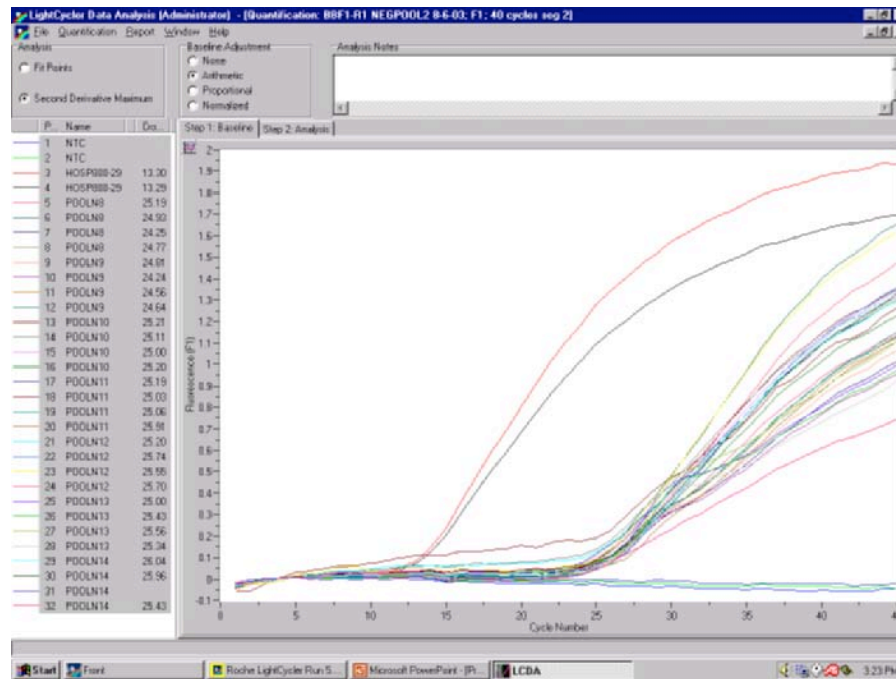


Figure 10. Pools of 10 negative *Lutzomyia verrucarum* show amplification, the NTC control was negative.

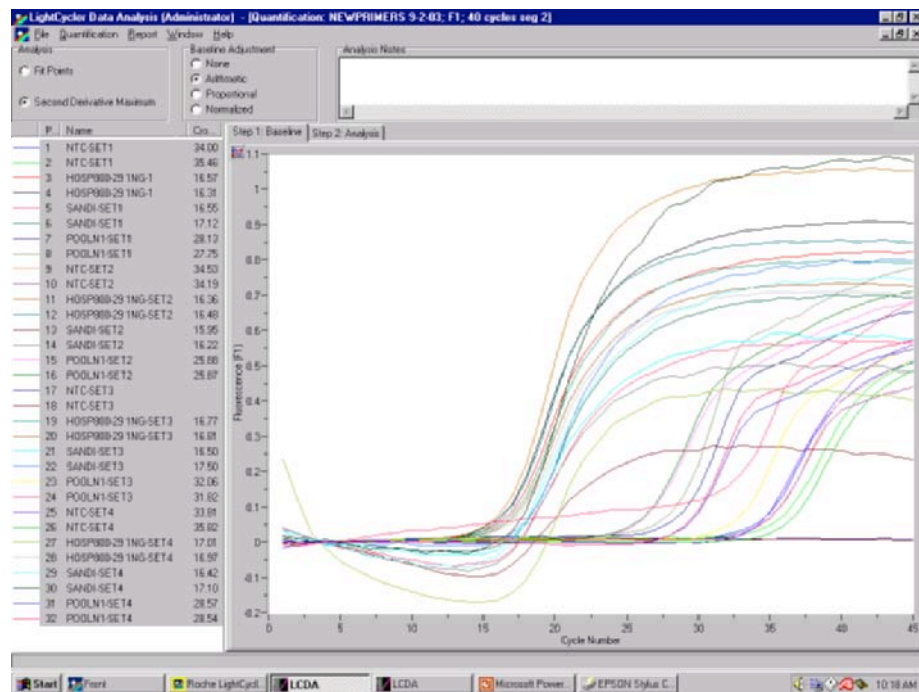


Figure 11. The first four sets of new primers were screened in one test.

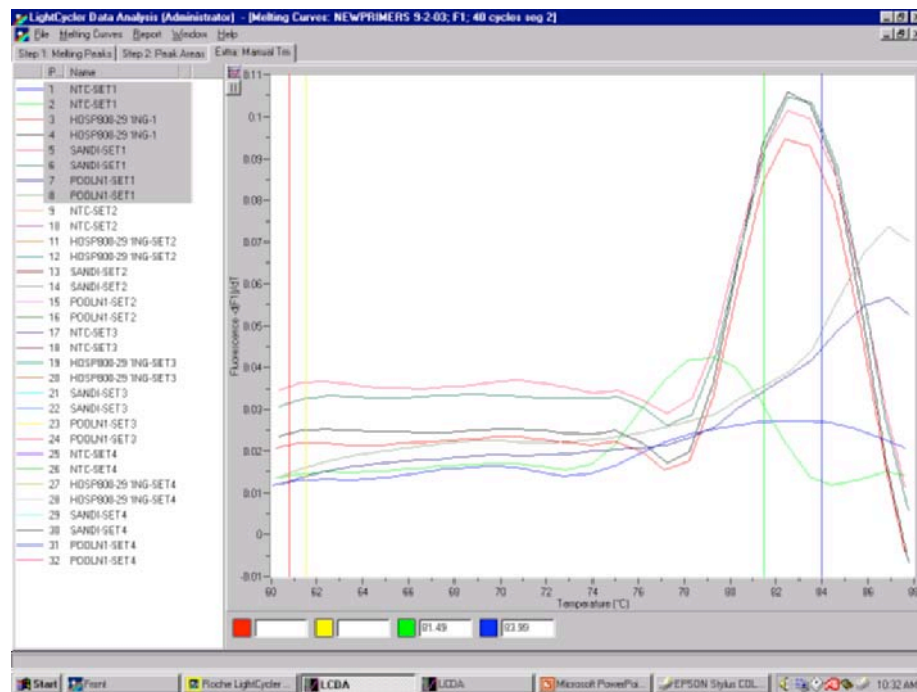


Figure 12. The melting temperature of the positive samples was 83°C and the negative pool was 87°C using primer Set1.

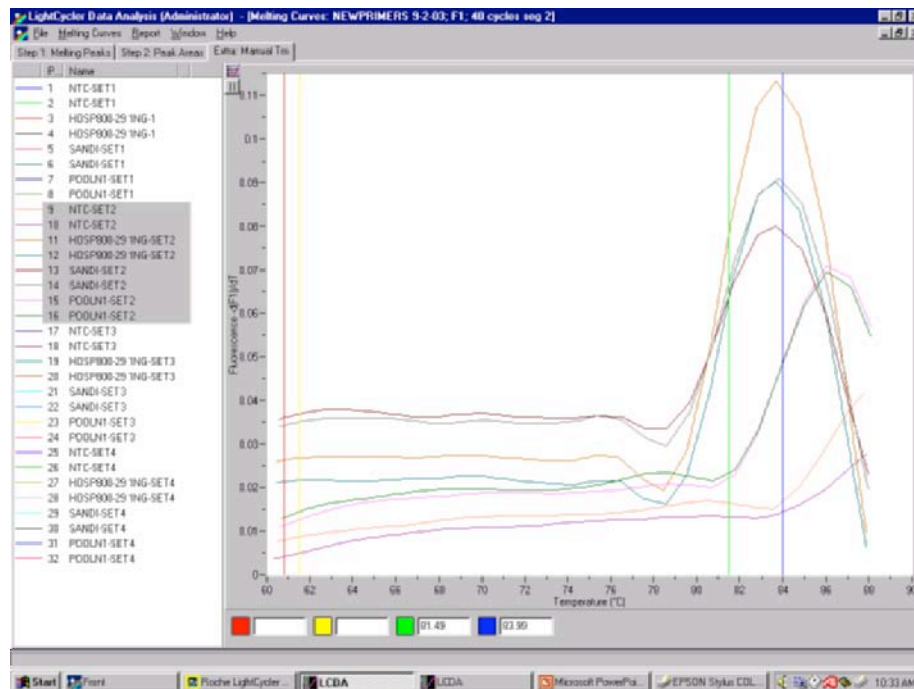


Figure 13. The melting temperature of the positive samples was 84°C and the negative pool was 86°C using primer Set2.

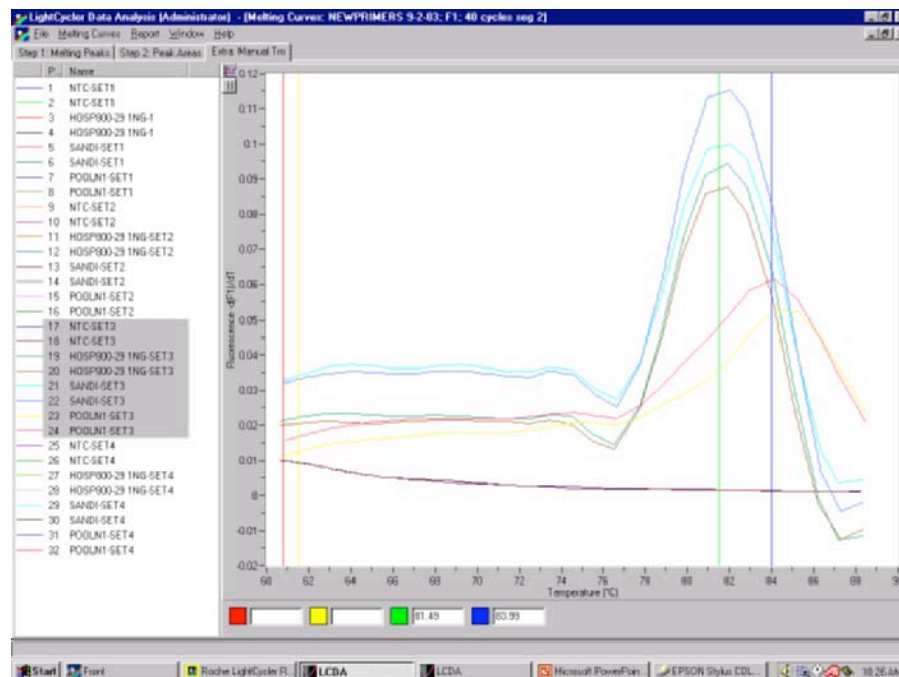


Figure 14. The melting temperature of the positive samples was 82°C and the negative pool was 84°C using primer Set3, NTC had no amplification.

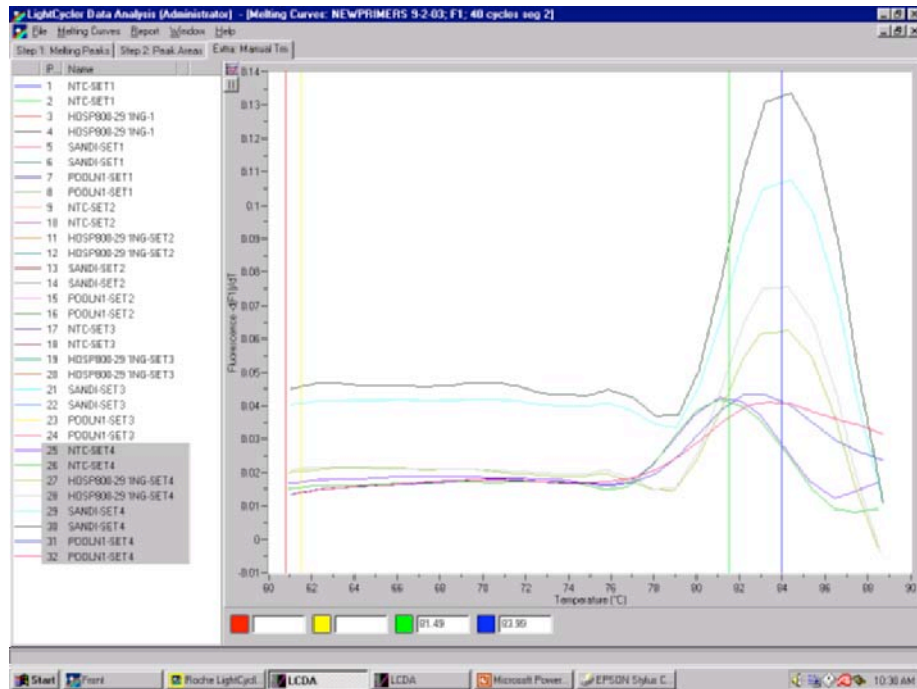


Figure 15. The melting temperature of the positive samples was 84°C and the negative pool was 82.5°C using primer Set4.

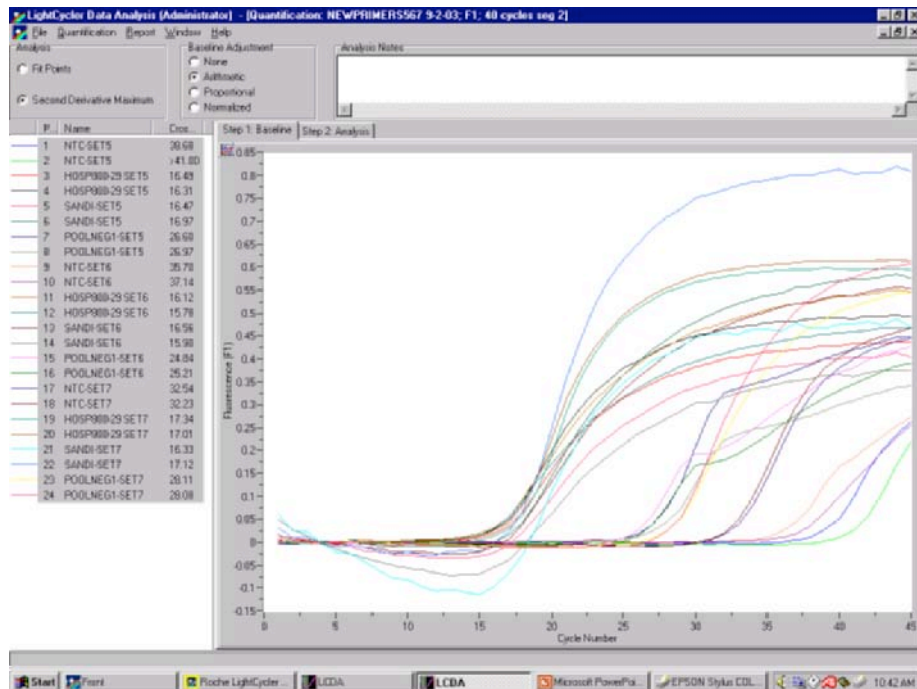


Figure 16. Last three sets of primers showed less fluorescence.

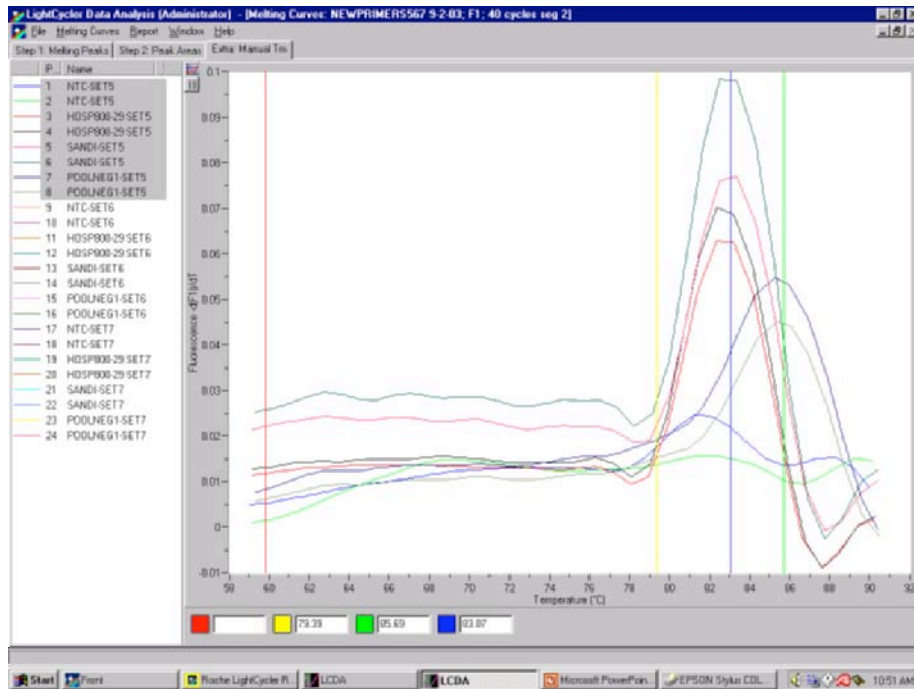


Figure 17. The graph shows the melting temperature of the positive samples was 83°C and the negative pool was 86°C using primer Set5.

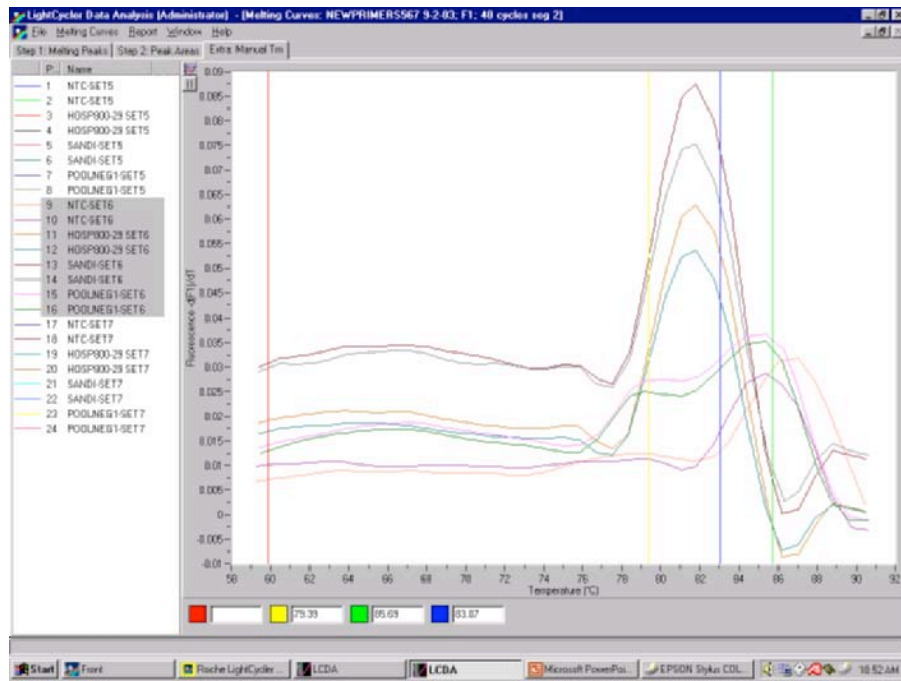


Figure 18. The melting temperature of the positive samples using primer Set4 was 82°C, the negative pool showed double peaks of 79 and 85°C.

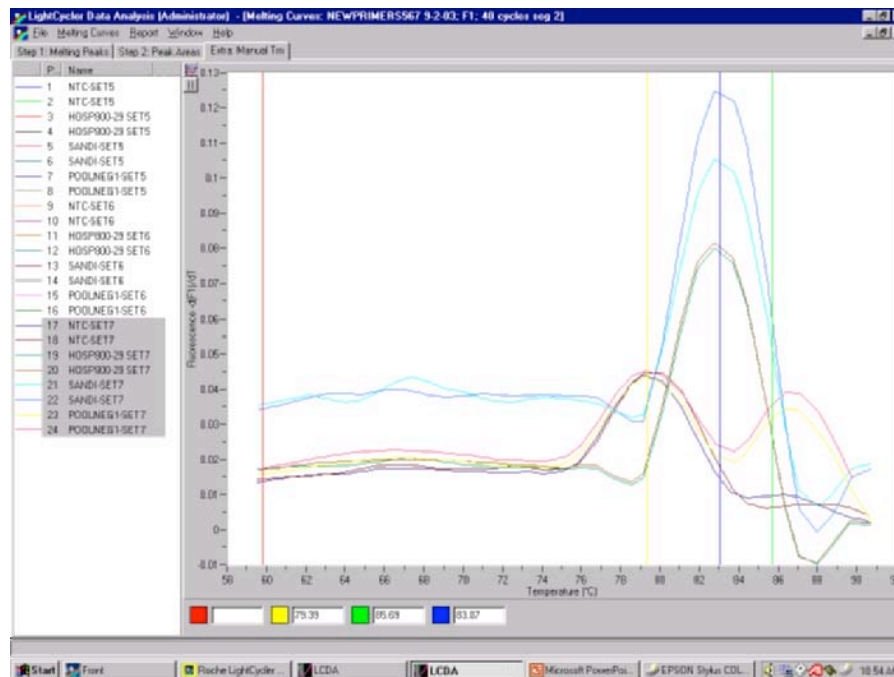


Figure 19. The graph shows the melting temperature of the positive samples was 83°C and the negative pool showed 2 peaks of 79 was 87°C using primer Set7.

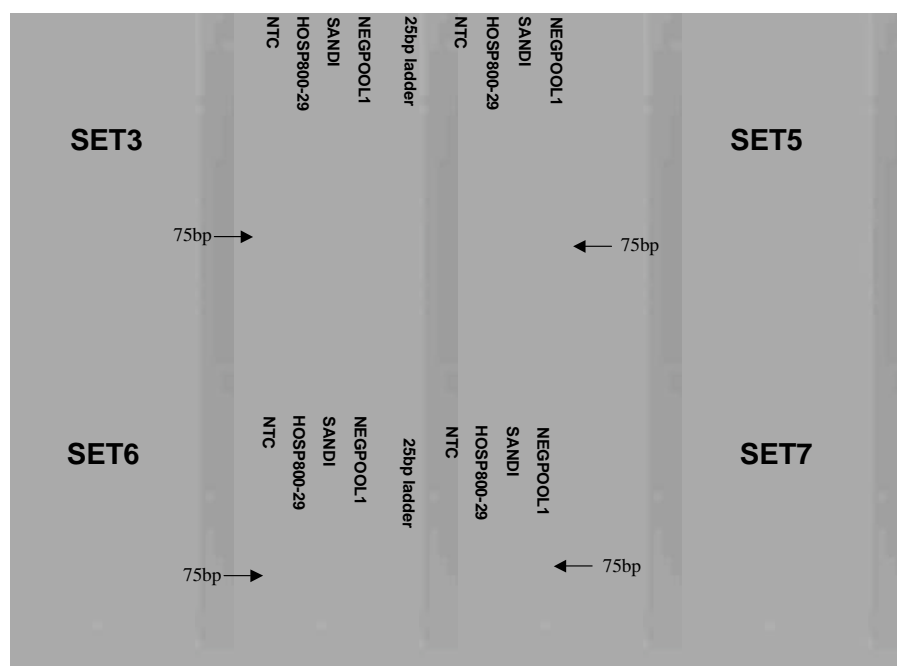


Figure 20. Gel electrophoresis showed that all the primers amplified multiple bands for the negative sand fly pool.

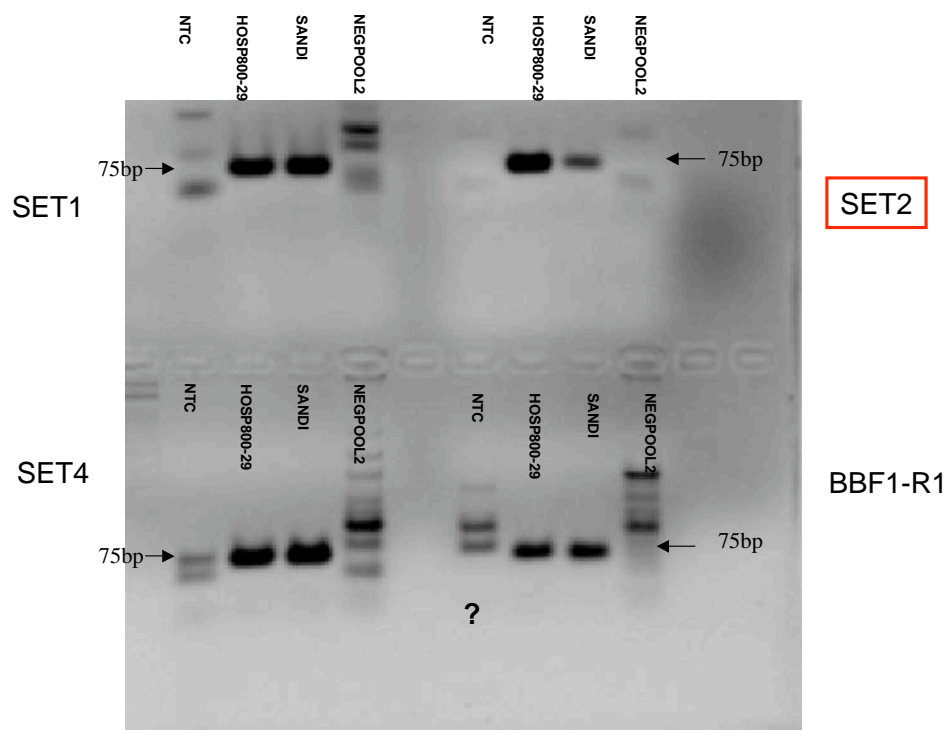


Figure 21. Primer set 2 had the same band pattern for the NTC and the negative sand fly pool and no 75bp band.

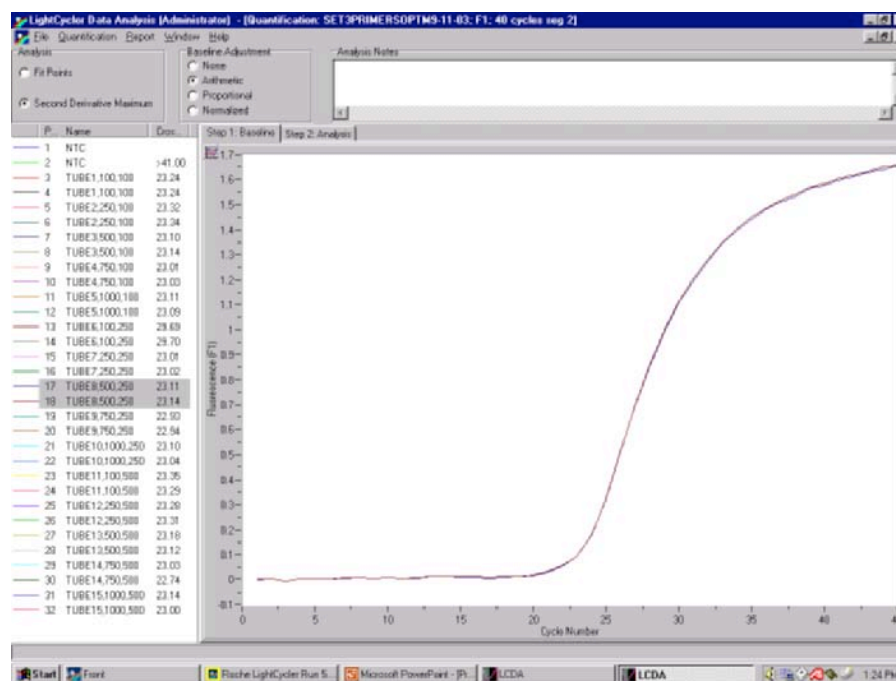


Figure 22. Tube 8 with 500nM for the forward and 250nM for the reverse had the lowest CT and the highest fluorescence.

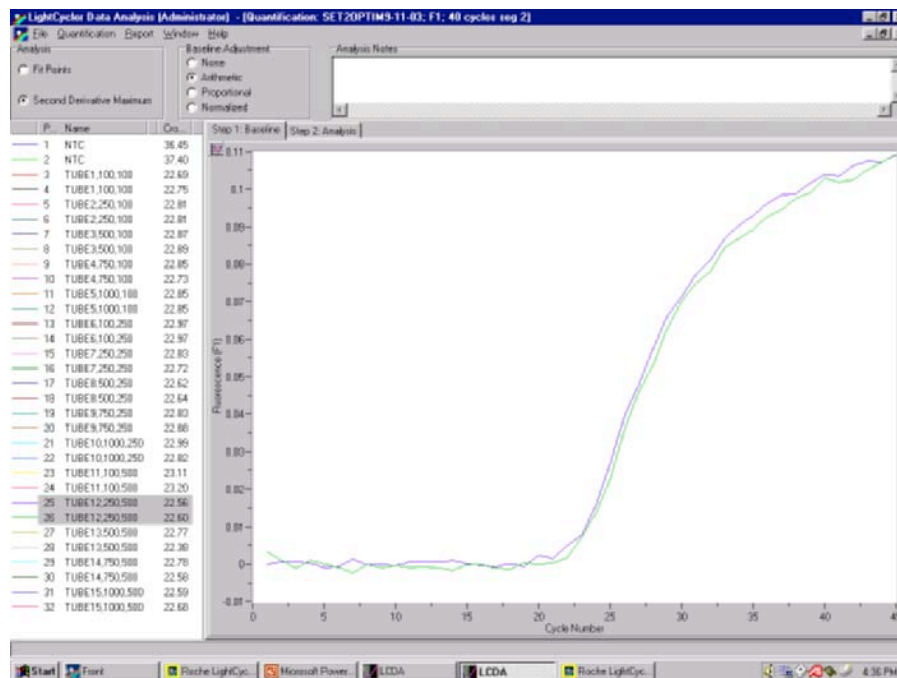


Figure 23. Tube 12 with 250nM for the forward and 500nM for the reverse was selected as the optimal primer dilution.

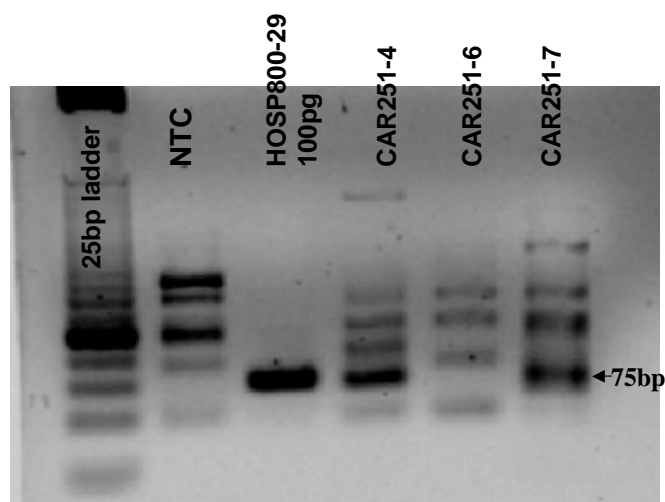


Figure 24. Gel electrophoresis of field samples were tested using Set2 primers with Sybr Green

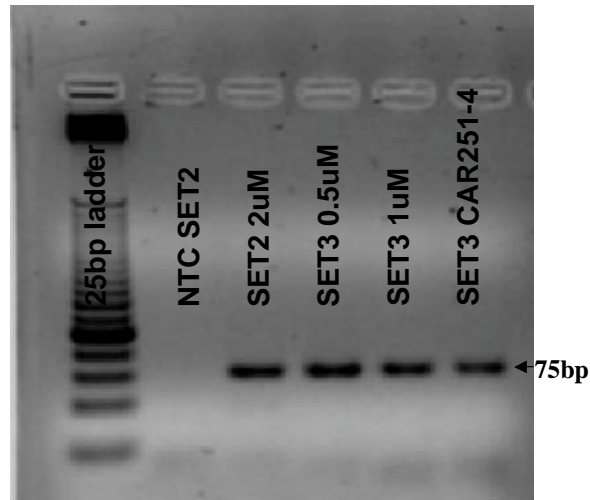


Figure 25. New probes B2-203 and BB3-543 were screened at different concentrations along with some field samples.

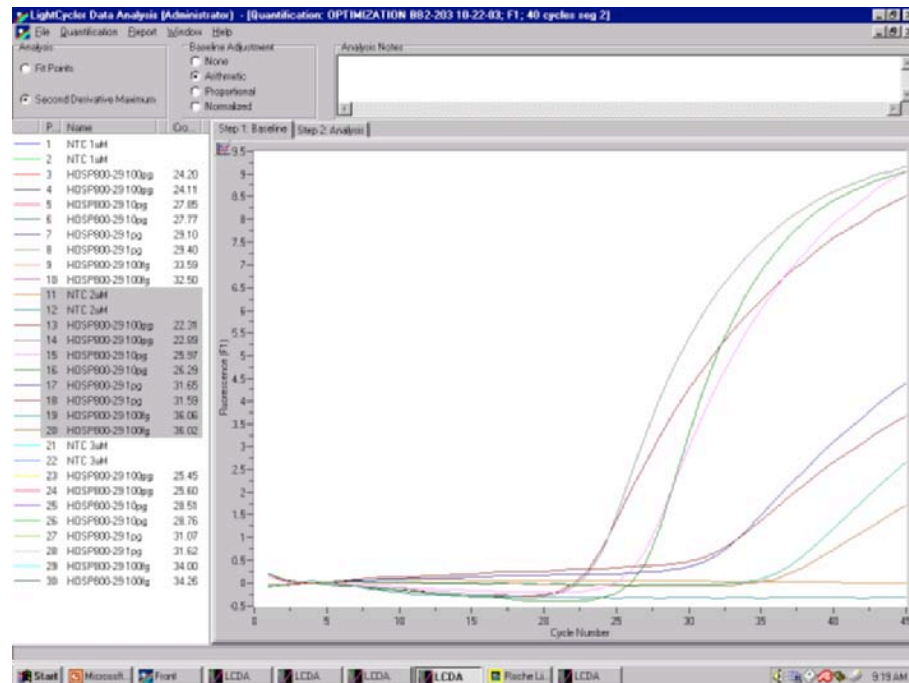


Figure 26. Optimization of Probe BB2-203 showed that working stock of 2\_M gave a higher fluorescence and lower CT's.

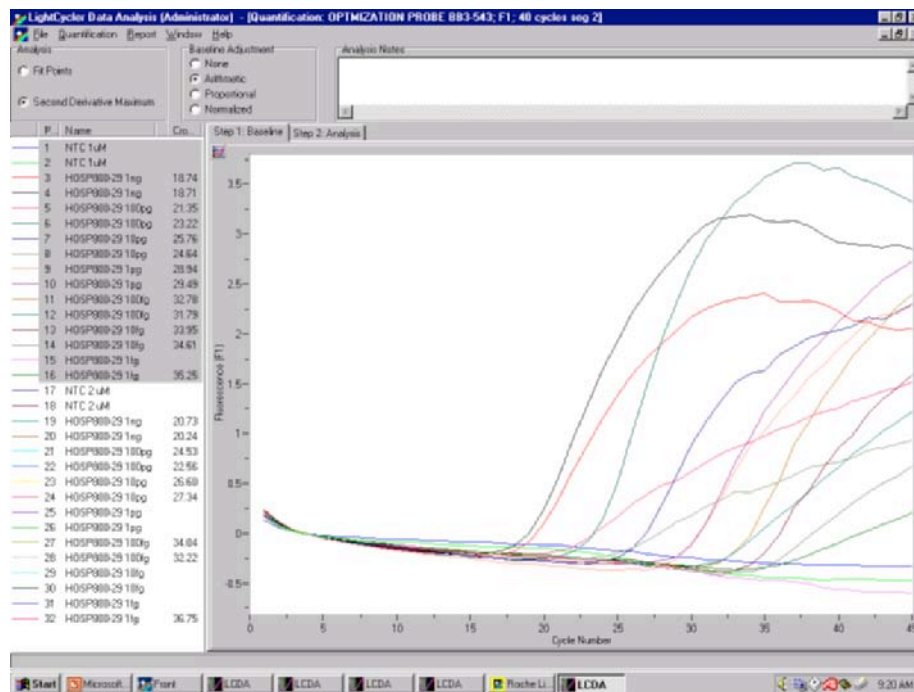


Figure 27. Optimization of Probe BB3-543 showed that working stock of 1\_M gives a higher fluorescence and lower CT's.

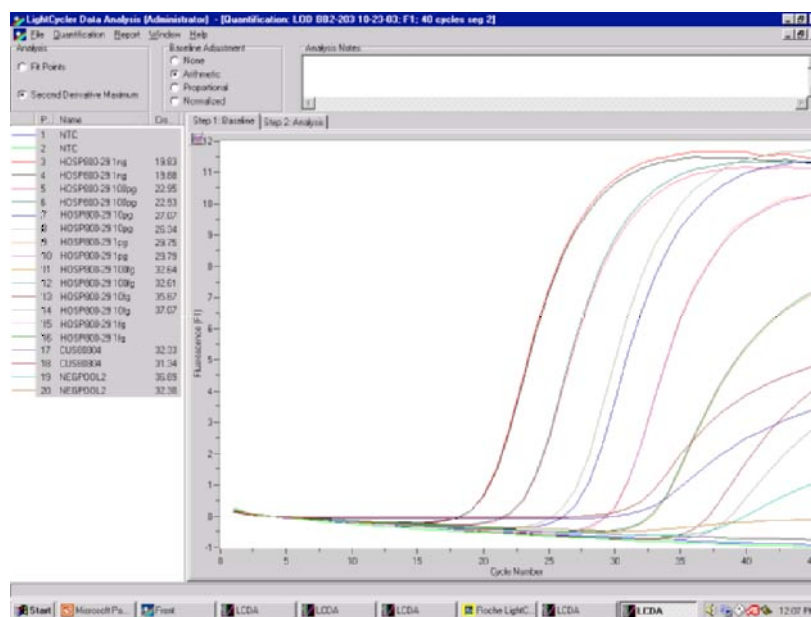


Figure 28. Limit of detection quantification was positive up to 10 fg/5\_l dilutions.

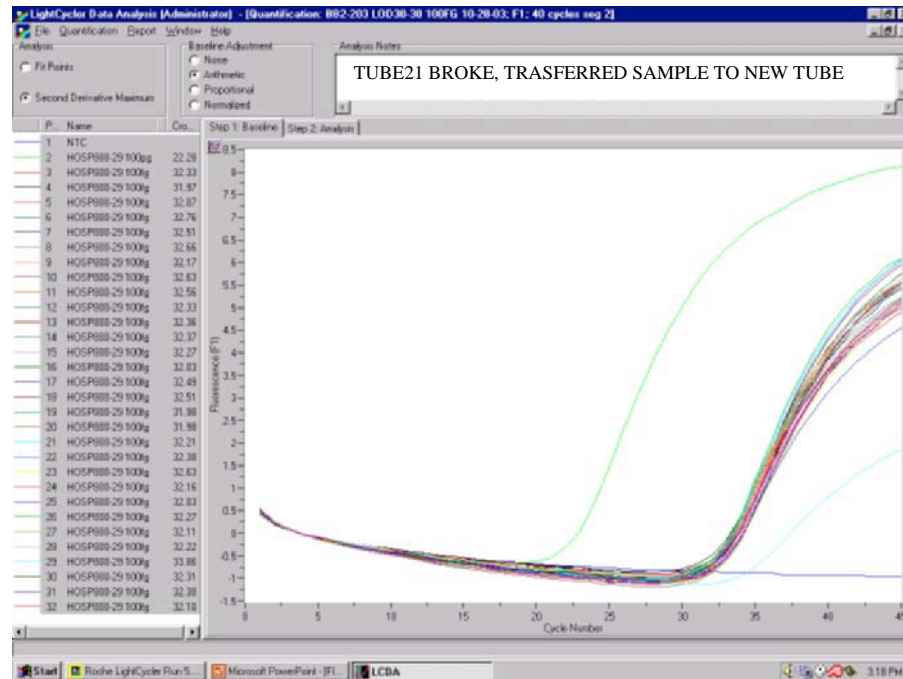


Figure 29. The LOD was 100fg/5µl when using probe BB2-203.

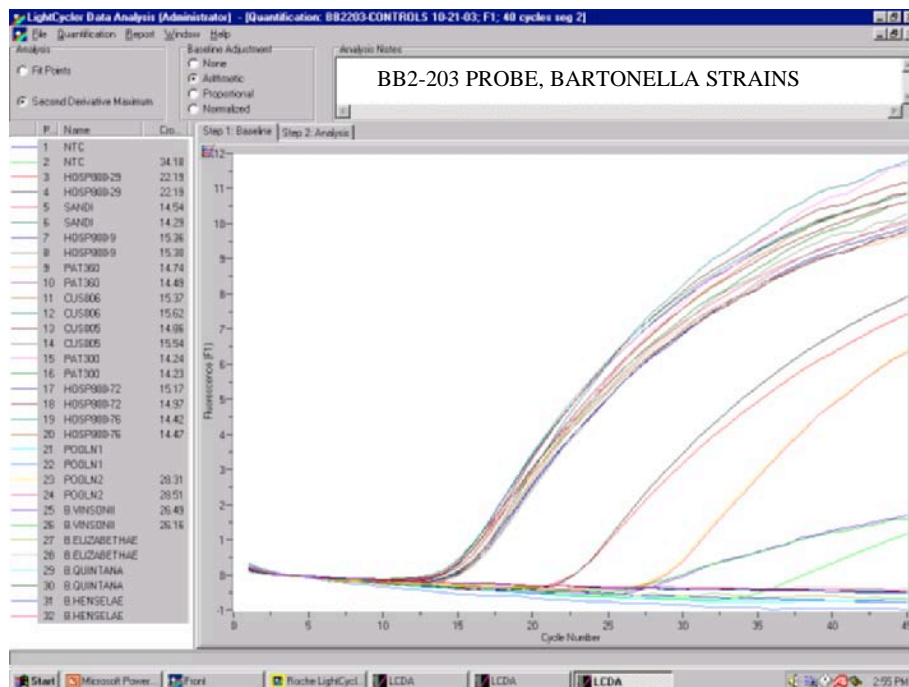


Figure 30. Specificity test for Probe BB2-203.

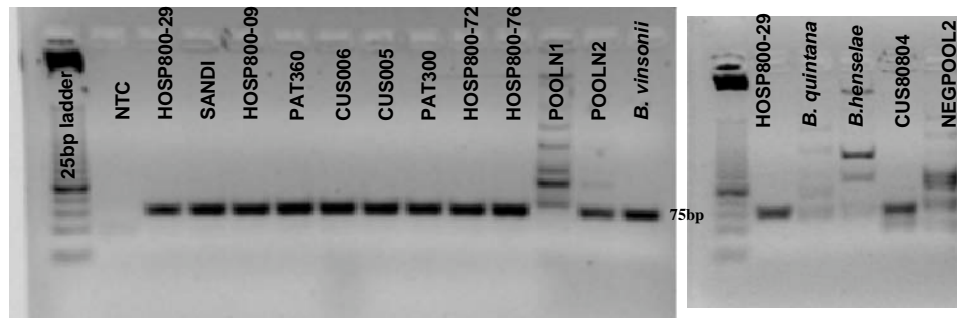


Figure 31. *Bartonella* strains and “Negative” pools

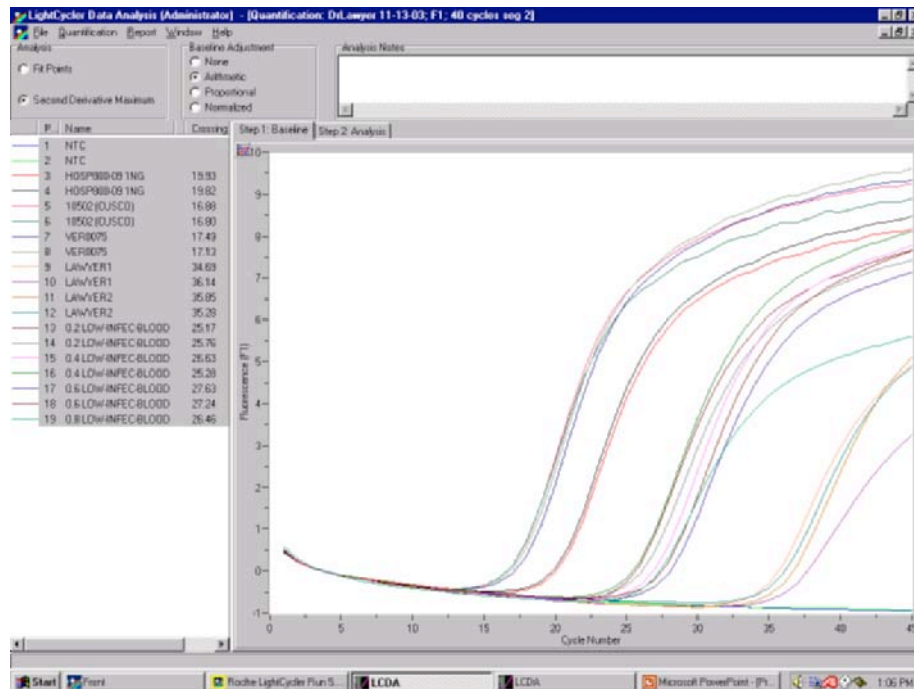


Figure 32. Strain controls, different volumes of low infected red blood cells and P.L.'s blood were amplified.

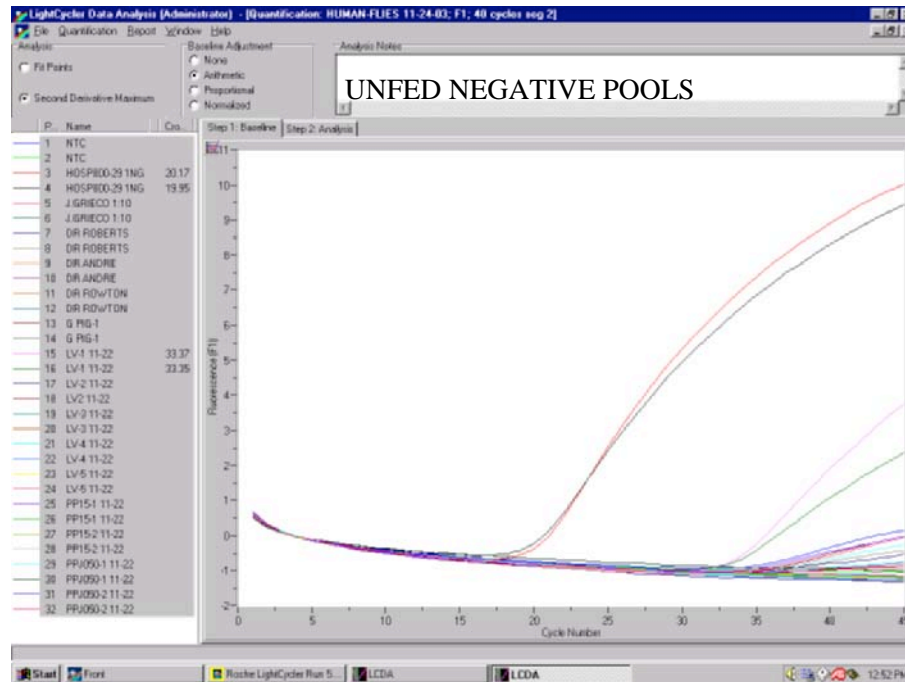


Figure 33. Sand flies genes were not cross-reacting with the test.

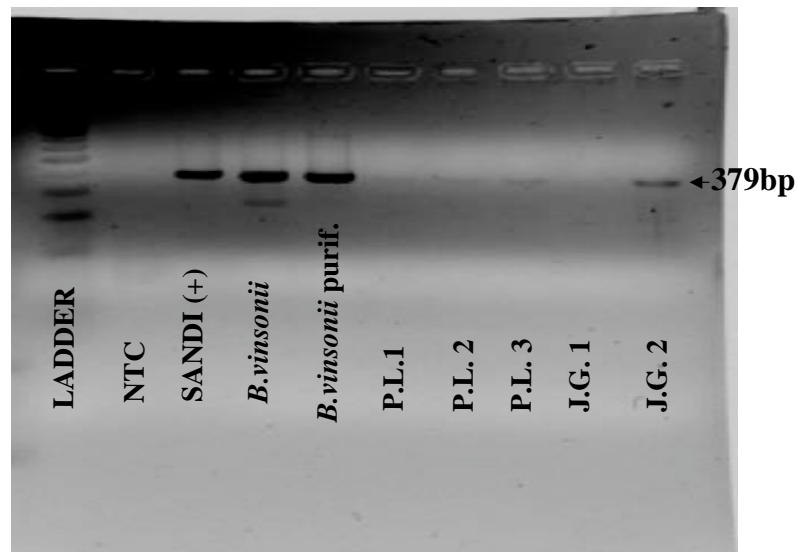


Figure 34. Conventional PCR using 781P, 1137N primers showed a 379bp band in the positive samples.

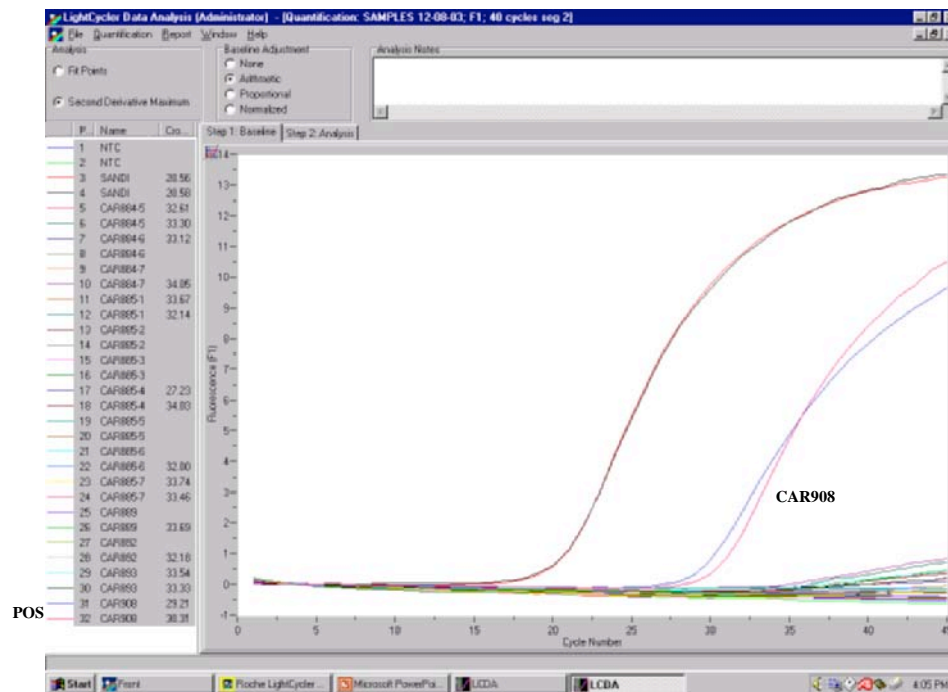


Figure 35. Few field samples gave high positive, many samples with CT readings were possibly negative, due to the low fluorescence.

#### PRIMER SET 2

```

1 ttatatcgat ggagatgaag gaatattact ttaccacgga tattctattg accaattagc
61 tgaaaaatgga gattttcttg aaacatgcta ccttttgctt tatggtgagt tgccaaacaa
121 acaacaaaaa atagattttg atcgctgtat tatgcggcat acaatgggtc atgaaacaatt
181 tgcacgcttc ttccacggat ttggcggcga ttctcactct atggtgtgta tggcgtcttg
241 tcttgggtgct atgtctgcat tttatcatga ctctattaat attacagatc ctcaacagag
301 aatgattgcc tctattcgcc ttatctcaaa gggtccaact cttgctgcta tggcatataa
361 ataatgattt gggaacactt ttgtttatcc acgtaatgac ctttaattacg ctacaaaattt
421 tcttcatatg tgcttctctg ttcttctgtg agaacacaaa attagccctg ttattgctcg
481 agctatggat cgaatcttta ctcttcacgc agatcatgaa caaaatgcac ctacgtcaac
541 agtacgcctt gcagggttcat caggagctaa tccgtatgca tgtattgcag cagggtgttg
601 atgcctttgg ggaccagctc atggtggagc taatgaagca tgtctaaaaa tgcataaaga
661 aatagggttct gttaaaaaaa ttcttgaatt tattgcgcgt gcaaaagata aaaatgatcc
721 ttttctctct atgggcttcg gccacagagt ctacaaaaat tatgatccac gtgcaaaaat
781 tatgcagaaa acctgccatg aagttttaca agagctcaac attcaagatg acccacttct
841 tgatatagcg atggagcttg aacacatcgc tctgaatgat gaattattca tcaacaaaaa
901 gctttatcct

```

Primer BB175  
acaatt tgcacgcttc ttcca

Primer BB249R  
tggcgtcttg tcttgggtgc  
accaggaac agaaccacg ←

PROBE BB2 203  
6FAM tct cgc gat tet cat cct atg gct gtc TAMRA

75 bp products

Figure 36. Primers and probes distributed in the citrate synthase gene.

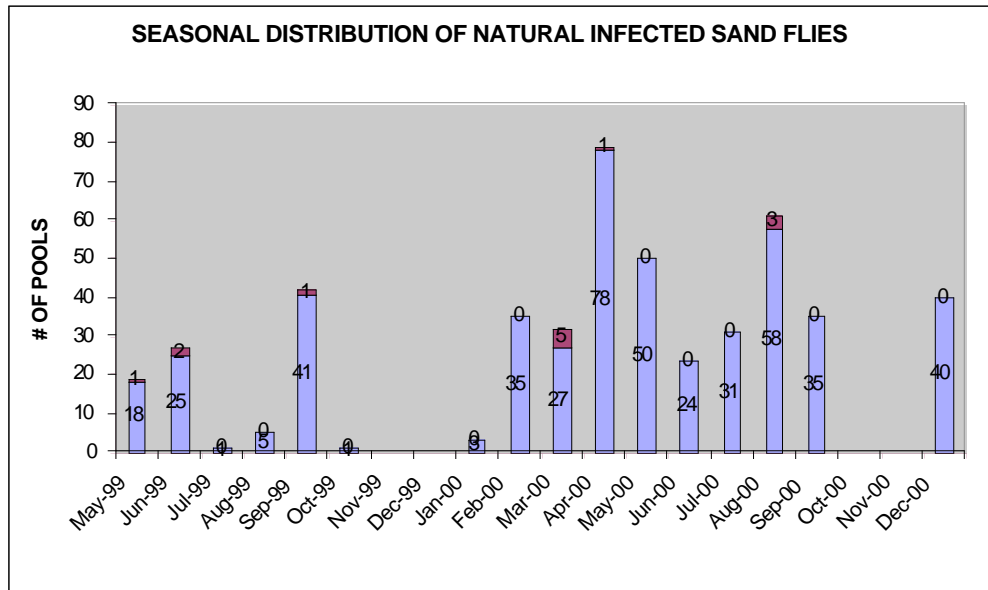


Figure 37. Season distribution of all the pools tested in 4 towns of Peru.  
Shaded area represents positive pools to PCR.

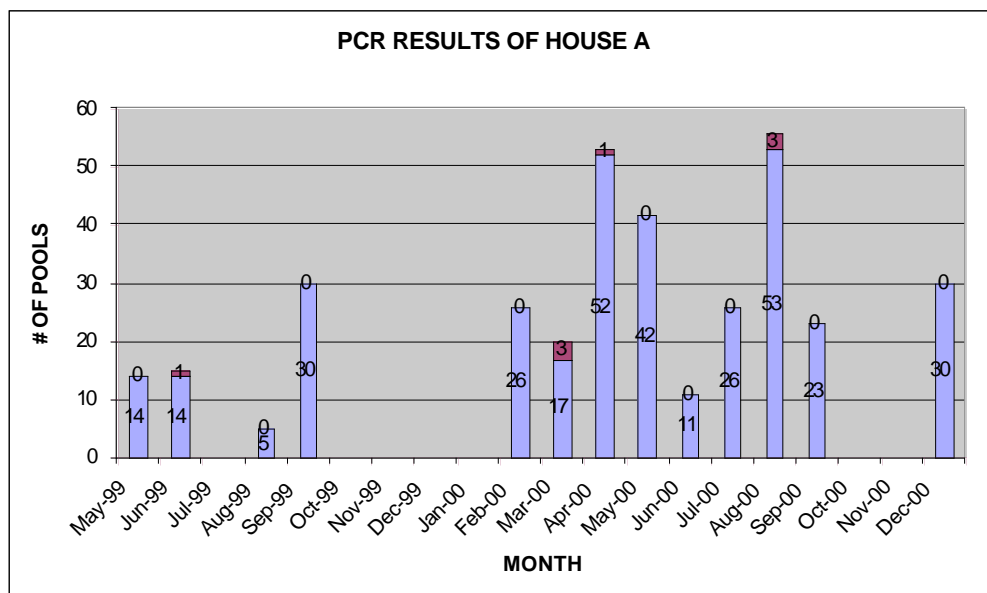


Figure 38. Season distribution of infected pools tested in house A.  
Shaded area represents positive pools to PCR.

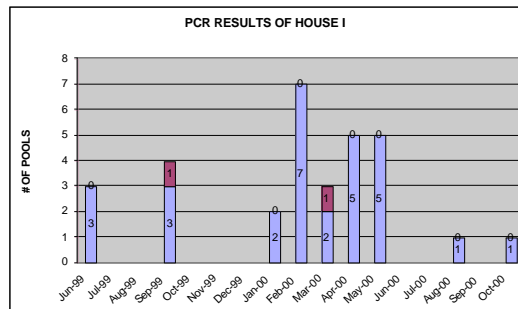
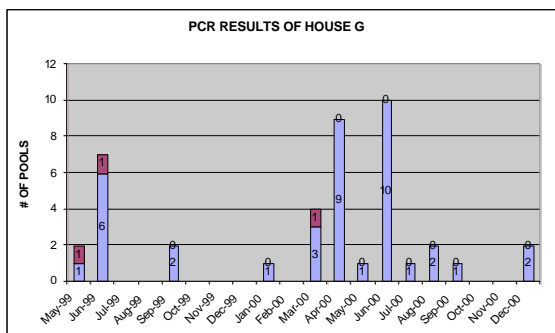
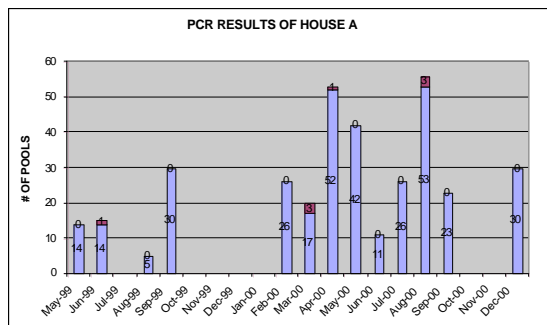


Figure 39. Season distribution comparison of infected pools of positive houses.  
Positive pools were found in all houses in samples of March 2000.

## BIBLIOGRAPHY

- Alexander, B. 1995. A review of bartonellosis in Ecuador and Colombia, Am. J. Trop. Med. Hyg. 52:354-359.
- Alexander B. and Marolli M. 2003. Control of phlebotomine sandflies. Med Vet Entomol. Mar; 17(1): 1-18.
- Barbian K.D. and Minnick M.F. 2000. A bacteriophage-like particle from *Bartonella bacilliformis*. Microbiology 146:599-609.
- Battisti, J.M. and Minnick, M.F. 1999. Development of a System for Genetic Manipulation of *Bartonella bacilliformis*. Appl. and Environ. Microbiol. 65:3441-3448.
- Battistini, T. 1925. Contribución al estudio de la verruga peruana. Nota preliminar. Anales de la Facultad de Medicina (Lima) (Número extraordinario) 1925: 27 - 33.
- Bejarano EE, Sierra D. and Velez ID. 2003. New findings on the geographic distribution of the verrucarum group (Diptera: Psychodidae) in Colombia Biomedica. Sep; 23(3):341-50.
- Benlarbi M. and Ready PD. 2003 Host-specific *Wolbachia* strains in widespread populations of *Phlebotomus perniciosus* and *P. papatasi* (Diptera: Psychodidae), and prospects for driving genes into these vectors of Leishmania. Bull Entomol Res. Oct; 93(5):383-91
- Bereswill S, Hinkelmann S, Kist M. and Sander A. 1999. Molecular analysis of riboflavin synthesis genes in *Bartonella henselae* and use of the *ribC* gene for differentiation of *Bartonella* species by PCR. J Clin Microbiol. Oct; 37(10):3159-66.
- Birtles RJ and Raoult D. 1996. Comparison of partial citrate synthase gene (*gltA*) sequences for phylogenetic analysis of *Bartonella* species Int J Syst Bact, Vol 46, 891-897
- Buckles EL, McGinnis and Hill E. 2000. Interaction of *Bartonella bacilliformis* with human erythrocyte membrane proteins. Microb Pathog. Sep; 29(3): 165-74.
- Caceres AG. 1993. Geographic distribution of *Lutzomyia verrucarum* (Townsend, 1913) (Diptera, Psychodidae, Phlebotominae), vector of human bartonellosis in Peru Rev Inst Med Trop Sao Paulo. Nov-Dec; 35(6): 485-90
- Caceres AG, Galati EA, Le Pont F. and Velasquez C. 1997. Possible role of *Lutzomyia maranonensis* and *Lutzomyia robusta* (Diptera: Psychodidae) as vectors of human bartonellosis in three provinces of region nor Oriental del Maranon, Peru. Rev Inst Med Trop Sao Paulo. Jan-Feb; 39(1): 51-2

Caceres-Rios H, Rodriguez-Tafur J, Bravo-Puccio F, Maguina-Vargas C, Diaz CS, Ramos DC. and Patarca R. 1995. Verruga peruana: an infectious endemic angiomatosis. Crit Rev Oncog. 6(1): 47-56.

Chamberlin, J., Laughlin, L., Gordon, S., Romero, S., Solorzano, N. and Regnery, R. 2000. Serodiagnosis of *Bartonella bacilliformis* infection by indirect fluorescence antibody assay: Test development and application to a population in an area of bartonellosis endemicity. J. of Clinical Micro. 38:4269-4271.

Chamberlin, J., Laughlin, L., Romero, S., Solorzano, N. Gordon, S., Andre, R, Pachas, P., Friedman, H., Ponce, C., and Watts, D. 2002 Epidemiology of endemic *Bartonella bacilliformis*: A prospective cohort study in a Peruvian mountain valley community. J. of Infectious Diseases. 186:983-90.

Coleman, S.A. and Minnick, M.F. 2001 Establishing a Direct Role for the *Bartonella bacilliformis* Invasion-Associated locus B (*Ia1B*) protein in human Erythrocyte Parasitism. Infect. Immun. 69:4373-4381.

Cuadra M. and Takano J. 1969. The relationship of *Bartonella bacilliformis* to the red blood cell as revealed by electron microscopy. Blood 33(5):708-715

Ellis B.A., Rotz L.D., Leake J.A., Samalvides F., Bernable J., Ventura G., Padilla C., Villaseca P., Beati L., Regnery R., Childs J.E., Olson J.G. and Carrillo C.P. 1999. An outbreak of acute bartonellosis (Oroya fever) in the Urubamba region of Peru, 1998. Am. J. Trop. Med. Hyg. 61:344-349.

Goldenthal, M J., Marin-Garcia, J. and Ananthakrishnan R. 1998. Cloning and molecular analysis of the human citrate synthase gene. Genome, 41 (5): 733-8

Gordon S, Andre R, Lawyer P, Fernandez R, Solorzano N, Marston E, Regnery R, Watts D and Laughlin L. 2000. Surveillance for *Bartonella bacilliformis* in biting arthropods from an endemic focus and the site of a recent epidemic in the Peruvian Andes. Joint Meeting of R. Soc. Trop. Med. Hyg., Brit. Soc., Parasitol. and Am. Soc. Trop. Med. Hyg. Oxford, England

Gray GC, Johnson AA, Thornton SA, Smith WA, Knobloch J, Kelley PW, Obregon Escudero L, Arones Huayda M and Wignall FS. 1990. An epidemic of Oroya fever in the Peruvian Andes. Am J Trop Med Hyg. Mar; 42(3):215-21.

Gupta RK, Robert LL and Lawyer PG. 2002. Arthropods of Military Importance. Kelley PK, Ed. Military Preventive Medicine: Mobilization and Deployment. In: The Textbook of Military Medicine. Washington, DC: The Borden Institute and The Surgeon General of the US Army, Wash., DC; pp 483-485

- Herrer A. and Christensen HA. 1975. Implication of *Phlebotomus* sand flies as vectors of bartonellosis and leishmaniasis as early as 1764. *Science*. Oct 10; 190(4210): 154-5
- Hertig, M. 1942. *Phlebotomus* and Carrión's Disease. Supplement to *Am. J. Trop. Med.* 22: 1-64
- Hertig M. and Fairchild, G.B. 1948. The control of *Phlebotomus* in Peru with DDT. *Am J Trop Med Hyg.* 28, 207-230.
- Houpikian P. and Raoult D. 2001. Molecular phylogeny of the genus *Bartonella*: what is the current knowledge? *FEMS Microbiol Lett.* 2001 Jun 12; 200(1)
- Innis MA and Gelfand DH 1990. *Protocols*. Innis, Gelfand, Sninsky and White, eds.; Academic Press, New York.
- Johnson G, Ayers M, McClure SC, Richardson SE and Tellier R. 2003. Detection and identification of *Bartonella* species pathogenic for humans by PCR amplification targeting the riboflavin synthase gene (*ribC*). *J Clin Microbiol.* Mar; 41(3):1069-72.
- Killick-Kendrick, R. and Ward, R.D. 1981. Ecology of the *Leishmania*. *Parasitol.* 82: 143-152.
- Knobloch, J., Solano, L., Alvarez, O. and Delgado, E. 1985. Antibodies to *Bartonella bacilliformis* as determined by fluorescence antibody test, indirect haemagglutination and ELISA. *Trop. Med. Parasitol.* 36:183-185
- Kosek M, Lavarello R, Gilman R.H., Delgado J., Maguina C., Verastegui M., Lescano A.G., Mallqui V., Kosek J.C., Recavarren S. and Cabrera L. 2000. Natural history of infection with *Bartonella bacilliformis* in a nonendemic population. *J. Infect. Dis* 182:865-872
- Kreuzer KA, Bohn A, Lass U, Peters UR and Schmidt CA. 2000. Influence of DNA polymerases on quantitative PCR results using the TaqMan probe format in the LightCycler instrument *Mol and Cell Probes*; 14:57-60.
- Latorre C.R., Rogers E., Romero S.E., and Fernandez R. 1989. Recovery of *Bartonella Bacilliformis* from cryopreserved *Lutzomyia verrucarrum* and *L. peruensis* (Diptera: Psychodidae) 38th Annual Meeting of the American Society of Tropical Medicine and Hygiene. 10-14 Dec. Honolulu, Hawaii.
- Lawyer PG and Perkins PV, 2000. Leishmaniasis and Trypanosomiasis. Eldridge BF & Edman JD eds. *Textbook of Medical Entomology*, Kluwer Academic Publishers, Boston, pp 231-298.

Maguiña, C. 1998. Bartonellosis o Enfermedad de Carrion, nuevos aspectos de una vieja enfermedad. A.F.A. Editores Importadores. Lima, Peru

Malqui, V., Speelman, E., Verastegui, M., Maguina, C., Pinell-Salles, P., Lavarello, R., Delgado, J., Kosek, M., Romero, S., Arana, Y., and Gilman, R. 2000. Sonicated diagnostic immunoblot for bartonellosis Clin Diagn Lab Immunol 7:1-5

McPherson, M. J. and Moller S.G. 2000. PCR. Springer-Verlag New York.

Norman, AF, Regnery, R., Jameson,P., Greene,C. and Krause, D.C. 1995 Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene J. Clin. Microbiol. 33 (7), 1797-1803

Pachas P. 2000 La Bartonellosis en el Peru. Oficina General de Epid. INS Modulo Tecnico Lima

Padmalayam I, Fiskus W, Massung RF and Baumstark BR. 2003. Molecular cloning and analysis of a region of the *Bartonella bacilliformis* genome encoding *NlpD*, L-isoaspartyl methyltransferase and *YajC* homologs. DNA Cell Biol. May;22(5):347-53

Rolain JM, Franc M, Davoust B, and Raoult D. 2003. Molecular detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. clarridgeiae*, *Rickettsia felis*, and *Wolbachia pipientis* in cat fleas, France. Emerg Infect Dis. Mar;9(3):338-42

Sobraques M., Maurin, M., Birtles, R. J., and Raoult, D. 1999. In vitro susceptibilities of four *Bartonella bacilliformis* strains to 30 antibiotic compounds. 43:2090-2092

Svobodova M, Votypka J, Nicolas L and Volf P. 2003. *Leishmania tropica* in the black rat (*Rattus rattus*): persistence and transmission from asymptomatic host to sand fly vector *Phlebotomus sergenti*. Microbes Infect. Apr;5(5):361-4

Tejada A, Vizcarra H, Perez J, Caceres A, Quispe J, Pinto J, Zorrilla V, Suarez A and Picon L. 2003. Estudio clinico epidemiologico de bartonellosis humana en el valle del Monzon, Huamalies, Huanuco. Anales Fac. Med. Univ. Nac. Mayor San Marcos Vol.64, (4):211-7.

Torgeson DG, Lampo M, Velazquez Y and Woo PT. 2003. Genetic relationships among some species groups within the genus *Lutzomyia* (Diptera: Psychodidae). Am J Trop Med Hyg. Nov;69(5):484-93

Trager, W and Jensen, J.B. 1976. Human malaria parasites in continuous culture. Science 193:673-675.

Velho PE, Cintra ML, Uthida-Tanaka AM, de Moraes AM and Mariotto A. 2003. What do we (not) know about the human bartonelloses? Braz J Infect Dis. Feb; 7(1): 1-6

Wu DY, Ugozzoli L, Pal BK, Qian J and Wallace RB. 1991. The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. DNA and Cell Biology 10 (3):233-238.

Zeaiter Z, Fournier PE, Greub G and Raoult D. 2003. Diagnosis of *Bartonella* endocarditis by a real-time nested PCR assay using serum. J Clin Microbiol. Mar; 41(3):919-25.

NIH web: <http://www.niehs.nih.gov/training/manuals/primerex.pdf>

MINSA (Ministry of Health, Peru):  
<http://www.minsa.gob.pe/infovigia/normas/bartoneiosis/capitulo01.htm>